

France and the nuclear illusion

Of all the damage that will be done by France's tests of nuclear weapons in the Pacific, the most serious is that it will complicate and perhaps undermine the negotiation of a comprehensive test-ban treaty.

AMONG modern democracies, France has an admirable record of consistency. Research programmes devised by one government are diligently followed by its successors for example. The same is true of French policy on nuclear weapons, which has hardly changed since General Gallois first spelled out, in the 1950s, the doctrine that countries such as France could defend themselves against powerful adversaries with relatively modest nuclear forces, provided they could inflict on the adversary damage commensurate with their own value as prizes of war. This is not the doctrine of deterrence in the classic Cold War sense, but an assertion that even modest nuclear powers are likely to enjoy greater immunity from attack than non-nuclear neighbours. From that has followed the *force de frappe* (based on Mirage aircraft) and now the missile submarines and air-launched cruise missiles whose warheads France hopes to test in the Pacific. Given the consistency of the record, President Jacques Chirac may be genuinely surprised at the fuss the testing programme has generated.

There are three components of the protests that should be clearly distinguished. First, there are environmental worries: an explosion may vent radioactivity to the atmosphere or, more seriously, the basalt core of the atoll in which the tests are planned may be so ruptured that radioactivity gets into the surrounding ocean. Almost certainly, the past few weeks will have ensured that those in charge take extra care, but it is also helpful that France has agreed that there should be a fact-finding visit by a group of experts representing the European Union (of which France is a member). If Chirac wished, as he should, to abate further fuss, he would agree that the testing programme should be held up until that group has had an opportunity to report. Nevertheless, the environmental hazard of the planned tests is probably as small as the French have been saying.

The second component of the recent fuss is less tangible but more important for France. In Australia, New Zealand and Japan, countries all committed to non-nuclear defence, there is deep resentment that the accidents of past colonialism should have given France the right to test weapons in the Pacific, which they are determined should live up to its name. France will come to regret the lasting damage done to its relationships with these countries. It is also understandable that people in France's own territories in the region have seen the past few weeks as an opportunity to protest at France's continued presence. The rough behaviour of *gendarmes* has exacerbated that problem, just as the

old-fashioned assertion of sovereign rights over the testing atoll has offended responsible governments in the region.

But the most serious complaint against French conduct is one that must also be levelled against China. Their behaviour will compromise the international resolve to sign a comprehensive test-ban treaty a year from now. Both countries have said that they will sign. France says that its tests are a necessary preliminary; China, as usual, has said nothing in public, but presumably is similarly motivated. The obvious difficulty is the precedent these testing programmes will provide for those whose adherence to the proposed treaty is necessary for its effectiveness, India and Pakistan for example. They can now argue that they, like China and France, should be allowed to perfect their technology before signing on the dotted line. Both countries have overlooked the chief purpose of the treaty, which is first to fossilize the technology of nuclear weapons and then to encourage its erosion.

This is a big responsibility for France to take on its shoulders, especially because the adversaries against which the nuclear programme was originally directed have recently become straw men. France has no better case than Britain for maintaining separate nuclear forces. France's offer to put its nuclear weapons at the service of its European partners is unlikely to be taken seriously until France can also suggest what purpose they could serve. But Chirac should set out to define in his own mind what the purpose would be; that way, he would discover that his nuclear ambitions are an expensive illusion, as are the British. □

Patenting nature now

The use of ingredients from the neem tree sharpens questions about patent laws.

FOR centuries, the neem tree (*Azadirachta Indica*), a tropical evergreen indigenous to the Indian subcontinent, has played a venerated role in the social life of the region. The chemical elements found in extracts from both the seeds and the bark of the tree have found a wide range of uses — from eliminating insecticides on farm crops to combating tooth decay — and their efficacy has been confirmed by numerous scientific studies, in both Indian and foreign laboratories. And the shade of the neem tree has frequently provided shelter for both formal and informal gatherings that



has enhanced its purely practical contributions to Indian life. Three years ago, the US National Research Council called its report on the remarkable properties of the tree's products *Neem, A Tree for Solving Global Problems*.

But the agricultural and medical properties of the neem extracts have increasingly been attracting the interest of international companies involved in agriculture, many of whom are keen to find new forms of pesticides that may cause less environmental damage than those discovered in the chemistry laboratory — and certainly carry a more benign image as a 'natural' product. Identifying the active ingredients through conventional scientific techniques, and incorporating the knowledge gained into products and processes aimed at the growing markets of the region, has often required substantial investment in research and development. And the companies choosing to make these investments have often chosen to protect this investment by taking out patents on their 'discoveries'.

Such actions, however, are now under fire. The use by Western-owned companies of a natural product whose properties have been known to indigenous communities for centuries has become a symbol for what critics describe as a form of 'genetic imperialism' — and as such, the focal point of a broader conflict about differing approaches to the ownership of knowledge about the natural world. Challenges are being launched in parallel in Europe and the United States on patents held by the multinational company W. R. Grace to various neem extracts that the company has successfully turned into marketable products (see page 95).

The sentiment behind the patent challenges is understandable. In the past, knowledge about the neem tree and the properties of substances derived from its seeds has been treated by native communities as a public good. In contrast, new knowledge, derived in the scientific laboratory and integrated into commercial products, becomes, through the patent system, a private commodity. While companies such as Grace will argue, with some justification, that this is merely the way in which the modern world works, it should hardly be surprised if the consequences of its actions should provoke the reaction that it has.

But is this sufficient for the patents to be revoked — as critics are now demanding? So far, their case is not persuasive. It is entirely correct that any patented discovery should be subject to rigorous scrutiny as to its genuine originality; but this does not, by definition, preclude patents that make use of earlier discoveries, whether patented or not. Similarly, if India wishes to demonstrate its vitality as a modern economy, it needs to embrace the essential role of the patent system, even in areas from which patents have traditionally been excluded. This is not to dismiss the legitimate concerns of those who claim that a lack of recognition by the patent system of indigenous (and largely unwritten) knowledge can discriminate unfairly against communities that have yet to embrace Western-style innovation. But the neem patents should be judged by the terms on which they were granted — not on the extent to which they conflict with traditional knowledge systems that have only a marginal role in the modern world. □

Save Down House

It will be a signal dishonour if Britain fails to save the house in which Darwin lived and wrote.

SHELLEY'S sonnet *Ozymandias* is a searing testament to the futility of remembrance. Shelley himself is buried near a pyramid raised to one Cestius, a Roman who would otherwise have been forgotten. Thomas Hardy later mused that even this posthumous remembrance would have faded were it not that the pyramid makes a convenient marker for the graves of the later romantics (Keats as well as Shelley). Like Hardy, Charles Darwin is buried in Westminster Abbey. The memories of both men live on in their writings, many of Darwin's in these pages, which points to another irony; without Darwin's influence on the likes of Thomas Huxley, the very existence of this journal would be in question.

If Darwin lives on in his literature, what is to become of his house? Down House, southeast of London, is suffering from more than half a century of neglect, which the Natural History Museum (NHM) in London would now rectify. The roof leaks and much of the interior needs renovation. Darwin's greenhouse is dilapidated, and the famous 'sandwalk' — laid down by Darwin as a place for thinking great thoughts — is now an overgrown woodland track.

How has it come to this? The Darwins bought the house in 1842, after which Darwin rarely strayed: he died there in 1882. In 1929, the family sold it to a wealthy surgeon and philanthropist, who then left it to the Royal College of Surgeons (RCS), after which the rot set in. Things began to look up only in January 1993, when the NHM took on a 99-year lease, and set about raising the £3.2 million needed to restore the house and update its facilities for visitors. So far, more than £0.5 million has come from private subscription, and the NHM has now bid for £2.4 million from the 'Heritage' fund created by the National Lottery.

It is a scandal that the future of Down House should depend on this dubious form of voluntary taxation. More worrying is the let-out clause in the lease allowing that if, by January 1996, the NHM has not raised the funds to guarantee future maintenance of the house, it can be returned to the RCS. Given its track record, the RCS would probably let Down House collapse. Far better that the NHM should ask for the extra required to buy the house outright. The surgeons have shown too little interest in half a century to be regarded as worthy owners.

Down House may be a far cry from *Ozymandias*' Shattered Wreck, but why should we even care to save it? If Darwin, like *Ozymandias* or Cestius, were remembered for nothing more than his mere existence, that argument would carry some weight. It is because so much of Darwin survives, and his influence continues to be felt, that Down House is more than a gravestone to its most famous resident. □

Monitoring mission gets go-ahead as scientists dispute N-test risks

London. The French government bowed to pressure from its European partners last weekend by agreeing to allow a group of experts to evaluate whether its nuclear testing programme in the South Pacific is causing serious environmental damage.

It did so at a time of division within the scientific community over the likely effects of future explosions. With another seven explosions planned, claims that additional tests could fracture the atoll, releasing radioactivity into the ocean, have received a mixed response from geologists and seismologists.

Roger Clark, a nuclear explosions seismologist at the University of Leeds in the United Kingdom, says the atoll, having endured more than 120 explosions over the past three decades, is in danger of breaking up. The possibility of the atoll collapsing from further nuclear explosions "cannot be ruled out", he says.

But Paul Richards, a researcher at Columbia University's Lamont-Doherty Earth Observatory in Palisades, New York, says there is "no basis or information" to support such a claim. "The strongest argument the French have to justify testing on the atoll is the perceived lack of adverse environmental consequences."

A senior British seismologist, who asked not to be named, also claims that Clark is being excessively pessimistic. French scientists, he says, have been monitoring the site's geology carefully. "I don't think the French want to destroy their main test site," he adds.

But Clark, who is also a spokesman for the Verification Technology Information Centre, says he is concerned that, while the French may be confident of the atoll's ability to absorb large stresses, just one minor fracture could lead to an unexpected event. "I would never dismiss the possibility of an unmapped feature in 300 square-kilometres of space," he says. "It is not impossible that the French may have missed something and the unexpected could happen."

Much to the chagrin of anti-testing scientists, the dispute between geologists coin-

cides with news that France is to permit European Union observers to examine its test facilities under Article 35 of the Euratom Treaty. But the French government has still not said whether it will suspend the tests pending the release of the observers' report.

France's nuclear testing programme was moved to the Pacific in 1966, after its regular

— resulted in the explosion sites being moved deeper into the atoll's basalt.

French officials continue to argue the tests pose no immediate risk to the atoll's geology. Scientists say the high temperatures in the vicinity of a nuclear explosion liquefy the surrounding rock, turning it into a glassy substance that insulates the remaining rock and any groundwater from possible contact with the explosion's radioactivity.

But Clark points to two radioactivity leakages that led to ground rupture after underground nuclear tests in the Nevada desert in the United States in the 1970s as evidence that the 'insulating theory' is not entirely watertight.

"High temperatures quickly vapourize limestone," says Clark. "This produces large quantities of carbon dioxide gas. If the material were ever to get over-stressed, as it did in the Nevada desert, the ground above might explode."

Clark is only one of several British scientists who have voiced concern about the region's fragile geology. Colin Summerhayes, director of the British government-funded Institute of Oceanographic Sciences, claims that underground nuclear explosions at Mururoa could generate undersea landslides that could in turn create tidal waves large enough to buffet nearby islands.

Jane Plant of the British Geological Survey is also sceptical of French assurances, and has called for the setting-up of an international scientific task force to investigate the area's geology.

None of the three scientific studies undertaken so far, however, has suggested that the atoll is close to collapse. Nor has any reported a risk of radioactivity leaking into the ocean. But no mission was allowed complete freedom to take samples.

The first study was published by a team of French geologists led by Haroun Tazieff, a former director of the French national scientific research centre. The group, which visited the Pacific in 1982, found significant but not harmful amounts of radioactivity in the environment, from past atmospheric tests.

Tazieff's team concluded that there was insufficient evidence to suggest radioactivity was leaking into the ocean through cracks in the basalt basement of the atoll. But Tazieff called for more studies to be carried out.

One year later, the French government gave a team of scientists from Australia, New Zealand and Papua New Guinea access to the atoll. The Atkinson Commission, as it came to be known, spent four days on Mururoa and concluded that parts of the ►

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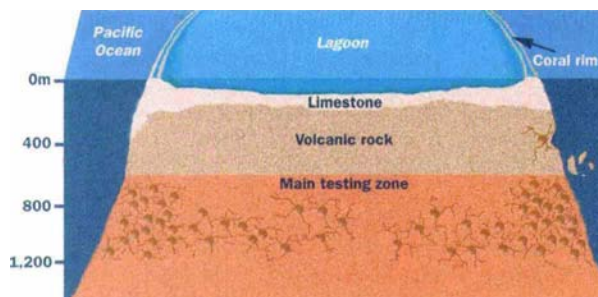
Shock wave: an official television picture of Mururoa's lagoon after last week's test.

site, Algeria, gained independence. Mururoa Atoll, an extinct volcano that has been stable for the past nine million years, was first choice. It was followed by Fangataufa atoll, 50 km away, where the French intend to test their more powerful explosions.

Mururoa is 1,200 km southeast of the island of Tahiti. A coral rim, three metres above sea level, encircles a natural lagoon 50 metres deep. The atoll is made up of variable depths of limestone, a transition zone, followed by volcanic rocks.

The atoll is filled with two-metre wide cavities from previous explosions that were carried out at depths of between 400 metres and 1.5 km. Computer simulations, says Clark, have shown that the large number of cavities close to each other make the atoll potentially unstable.

Until the 1980s, tests were conducted at shallow depths beneath the atoll's rim. But three instances in which explosions led to ground collapse — in 1977, 1979 and 1980



Mururoa Atoll forms the remains of an ancient volcano. It comprises mainly volcanic rock, covered by a thin layer of limestone coral. More than 120 nuclear explosions have caused only localized fractures. But one test, in the 1970s, blew away part of the volcano's side when a detonator became lodged in the shaft at 400m.

► atoll appeared to have been affected by localized fracturing around each test site, subsidence and submarine landslides. However, the scientists suggested radiation leakage would not occur for 500 to 1,000 years.

In 1987, the French marine biologist Jacques Cousteau conducted a further study of Mururoa. His report, released in 1988, reinforced the Atkinson observations. Cousteau also concluded that nuclear tests on the atoll posed neither short nor mid-term risks to the former volcano's geology.

Cousteau seems to have had a change of heart about the testing programme. He is now fervently anti-nuclear and has emerged as one of the French government's sternest critics, warning in an article in *Le Monde* last week of the dangers of undermining international efforts to reduce the dangers of nuclear non-proliferation.

Cousteau: tests carry serious political risks.

The data from Cousteau's 1987 study, meanwhile, were re-evaluated by Greenpeace scientists in 1990. Norm Buske, chief scientist on board their ship *Rainbow Warrior*, sampled zooplankton in waters just outside the 12-mile exclusion zone from Mururoa. Buske claims to have found traces of caesium-134, a known product of nuclear fission which has a half-life of two years, although any link with the underground tests is difficult to establish.

A paper prepared by Australian scientists for a meeting of environment ministers from the South Pacific last month (see *Nature* 376, 625; 1995) concluded that "it is unlikely there will be major rupturing of the atoll as a result of the remaining eight tests, although there is insufficient evidence to be absolutely certain". But the scientists described as "more significant" the risks of long-term leakage of longer-lived radioisotopes into the lagoon and surrounding ocean.

Ehsan Masood

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Rex Features

Nobel corruption claims are 'totally without substance'

Munich. The Nobel prize committee has angrily rejected accusations that one of its members accepted payments from the Italian drug company Fidia as part of a campaign by the company to promote the nomination of the Italian neuroscientist Rita Levi-Montalcini for the 1986 Nobel prize for physiology or medicine.

The accusations, which come only a month before this year's awards are due — and which have been described by the committee as "totally without substance" — were made in a series of three articles published last week in *Dagens Nyheter*, Sweden's largest daily newspaper.

In particular, the newspaper claimed that Tomas Hökfelt, a professor of neuroscience at Stockholm's Karolinska Institute, had accepted favours from Fidia. These included payment for his wife to join him at a Fidia-sponsored meeting in Madrid, and the acceptance of a Fidia prize worth US\$2,000 in 1986. The newspaper linked these with Hökfelt's support for Levi-Montalcini's nomination when he was a member of the Nobel prize committee.

Levi-Montalcini won the 1986 prize with the US researcher Stanley Cohen for her work on nerve growth factor in the 1950s. Her achievements opened up a broad new field of research on the mechanisms of nerve degeneration.

The Swedish newspaper says that it was not challenging the appropriateness of awarding the prize to Levi-Montalcini, nor making a personal attack on Hökfelt, but simply wanted to raise the question of whether the Nobel prize committee was potentially open to corruption.

The charges against Hökfelt are based on accusations — so far unproven — by Duilio Poggiolini, Italy's former head of drug regulatory affairs, that Fidia mounted a 14-billion lire (US\$8.7 million) campaign to

assure the prize for Levi-Montalcini, as her work had implications for its own commercial products, the gangliosides, which in the 1980s were candidate compounds for neurodegenerative disorders (see *Nature* 367, 672; 1994).

Poggiolini was arrested in 1993 on charges of corruption, and made the claim in court a few months later as part of his defence, arguing corruption was endemic. He is currently awaiting trial in Rome.

The newspaper's charges against Hökfelt arose from an examination of all his correspondence since 1982 — including thousands of letters — which the Karolinska Institute was obliged to release under Sweden's liberal freedom of information laws.

Sten Grillner, the chairman of the Nobel prize committee and professor of neuroscience at the Karolinska Institute, says he is appalled by the allegations against Hökfelt and the Nobel committee. "The newspaper has put together a story from unrelated facts that is completely without foundation."

Grillner claims that it would be impossible to corrupt the process of choosing Nobel prizewinners because of its complexity, and the fact that a large number of scientists is involved. "We also know that because the prize is so prestigious, it is always heavily scrutinized by the scientific public when it is announced," he says. "Up to now the prizes have been well-received."

He adds that he sees no reason why the selection process should be changed as a result of the accusations against the committee, adding that in his opinion all committee members are, by definition, of the highest integrity.

Hökfelt was the world's most cited neuroscientist in the 1980s, and Grillner says that both his position on the Nobel committee and invitations to scores of international meetings were due to his scientific eminence. It is entirely normal to accept expenses for attendance, he adds, and rejects the charge that this practice lays scientists open to corruption.

"If the Nobel prize committee were to prevent its members attending conferences with commercial sponsors, no-one would have attended any conference," says Grillner. Hökfelt has received at least ten scientific prizes, so the relatively small award he received from Fidia was not unusual, he says. "And if, as alleged, Fidia had mounted any campaign to influence the Nobel prize committee [in Levi-Montalcini's favour], we were certainly unaware of it," he says.

Hökfelt himself refuses to comment on the newspaper's charges, saying merely that the allegations against him "are totally unfounded".

Alison Abbott

Revised EC contracts improve property rights

London. The European Commission in Brussels has introduced a new model for research contracts that strengthens the protection of intellectual property rights awarded to researchers working on projects partly funded by the European Union's Fourth Framework Programme.

The new contracts give research consortia the legal right to ownership of intellectual property resulting from work on a research project, and also reinforce the confidentiality requirements regarding unprotected, unpublished scientific results.

The new model contract, which is half the length of the current model introduced in 1988, also clears up previous anomalies

in this area, and provides a framework for the commercial exploitation of technology. It also required the submission of an implementation plan at or before the end of the contract — a move intended to improve the exploitation of research results.

The new format has been introduced on the initiative of Edith Cresson, who was appointed commissioner for science, research and development at the beginning of this year, and Martin Bangemann, commissioner for industry, telecommunications and information technology.

The new model contract is the outcome of extensive consultations with industry, academia and research organizations. □

US physicists rally to save standards agency

Washington. The US science establishment has launched a campaign to save the National Institute of Science and Technology (NIST), which is looking increasingly vulnerable as Congress moves to close its parent agency, the Department of Commerce.

Twenty-five US recipients of the Nobel prize for physics wrote to Congressional leaders this week to emphasize the "essential role to the nation" of the NIST laboratories at Boulder, Colorado and Gaithersburg, Maryland. They were joined by the heads of eighteen major scientific groupings, led by the American Physical Society.

One of the Nobelists, Norman Ramsey of Harvard University, described NIST as "a great institution". He cited the first demonstration of Bose-Einstein condensation, at Boulder earlier this summer, as an example of the quality of the work of its laboratories.

A new bill aimed at abolishing the commerce department was introduced into the Senate last week by William Roth (Republican, Delaware), and passed through the Senate's government affairs committee, which he chairs.

The measure, which has considerable momentum in the upper house, would transfer the standards functions of NIST to a new Office of Patents, Trademarks and Standards. It makes no provision for the NIST laboratories, which employ around 3200 people in support of its standards work.

Many scientists are worried that Congress will overlook the laboratories as it rushes to close down the large extramural technology programmes which NIST also operates (see *Nature* 376, 453; 1995), and to dismantle the Department of Commerce, headed by the flamboyant former Congressman, Ron Brown. The department has emerged as the prime candidate for abolition in a Republican Congress that is keen to prove its radicalism by shutting down at least one government department.

A bill introduced into the House in June by Dick Chrysler (Democrat, Michigan) would transfer NIST's functions into the National Science Foundation (NSF). But critics point out that the NSF is a grant-giving body with no laboratories of its own, and no known desire to accrue any.

As well as the Bose-Einstein breakthrough, NIST laboratories have pioneered the development of magnetic microscopes, and standards and test methods for modern, digital communications networks. They also house the world's most accurate atomic clock, NIST-7, as well as the most heavily used neutron source in the United States, the Cold Neutron Research Facility at Gaithersburg.

So far this year, the US scientific establishment has concentrated most of its lobbying efforts on a hitherto successful effort to defend the budgets of the National Insti-

tutes of Health and the NSF, the two main grant-giving agencies in the federal government. As a result, Monday's plea by the Nobel laureates and scientific societies arrives perilously late in the day for NIST.

But scientific leaders are alarmed that proposals to disband the commerce department are about to be absorbed into a "reconciliation bill" which Congress will place on President Bill Clinton's desk in mid-November.

This vast bill will attempt to reconcile all federal spending and taxation. According to Michael Lubell, director of public affairs at the American Physical Society, NIST's \$250 million internal research programme could easily be "lost in the noise" surrounding it.

"As Congress prepares the omnibus reconciliation bill, its ignorance of NIST laboratory functions could easily result in actions that would cause irreparable harm to a vital government research activity, however unintended that harm might be," says Lubell.

Meanwhile a Senate appropriations subcommittee has approved a budget of \$223 million to run the NIST laboratories next year, compared to \$276 million proposed by

the House and \$311 million requested by Clinton. The Senate bill would also provide NIST with \$76 million to wind down its technology support programmes, against nothing from the House and Clinton's request of \$640 million to continue the programme.

Arati Prabhakar, the director of NIST, says the Senate figure for technology support is "totally unacceptable". Cuts in the laboratories' budgets will "pose some significant challenges," but are "not the sort of catastrophe" facing other programmes in the same bill, she says.

Prabhakar enthusiastically welcomed the scientists' expression of support for her laboratories. But the scientists have not been able to suggest a safe home for NIST if the commerce department is indeed shut down.

Robert Walker (Republican, Pennsylvania), chair of the House science committee, was set to put forward his proposed Department of Science as such a home at hearings on the Chrysler bill due this week. But Nobel laureate Robert Schrieffer of Florida State University says that the community is "close to unanimity" in rejecting that idea.

Scientific leaders add that there is no serious case to be made for a shotgun marriage with the NSF. The best hope for NIST now may be a period of uncomfortable limbo until the November 1996 presidential election. This is precisely what it will get if Congress abolishes the Commerce Department in a reconciliation bill which President Clinton then sees fit to veto. **Colin Macilwain**

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**Brown: destined to
lose his department?**

Neuroscientists seek meeting boost

Munich. The heads of 17 national neuroscience associations agreed last week to coordinate their activities more closely with those of the European Neuroscience Association (ENA), partly to raise attendance at ENA's annual meeting.

Between 15,000 and 20,000 participants — including several thousand European neuroscientists — regularly take part in the neuroscience meeting held every November in the United States and organized by the US Society for Neuroscience. In contrast, its European equivalent, held two months earlier, seldom attracts 2000 participants.

One major reason is cost; it is often cheaper to cross the Atlantic than to travel within Europe, and the large attendance at the US meeting means that registration fees can be kept low. But the European association also has to compete with national neuroscience meetings, which are also less expensive to attend.

To help solve both problems, the national bodies agreed in Amsterdam last week to study ways of moving towards a federal structure within ENA, and to coordinate the timing of their meetings with those of the

European body, to avoid direct competition.

According to Wolf Singer, head of neurophysiology at the Max Planck Institute of Brain Research in Frankfurt, and president of ENA, the European body proposed moving its own meeting from September to June to avoid clashing with the US meeting, starting in 1997 or 1998.

ENA — which is planning to approach the European Commission for financial support — suggests it should in future also hold its own meeting only every other year, alternating with national meetings. Singer says this is intended to increase the number of participants, and so reduce registration fees.

In turn, national societies' delegates have agreed to consider ways of coordinating their own meetings with this timetable. They will continue to meet on their own every two years, but will try to combine their meetings with that of the ENA in alternate years.

In contrast to the European body, national neuroscience organizations continue to attract relatively high numbers of participants. A final decision on the future structure of the ENA meetings will be made at its 1996 meeting in Strasbourg. **Alison Abbott**

Biosafety code gathers pace through bilateral agreements

London. Argentina last week became the first developing country to adopt internationally agreed safety guidelines covering the transportation and use of genetic engineering products that are being developed under the auspices of the United Nations Environment Programme (UNEP).

The move took the form of a bilateral agreement signed on 8 September in Buenos Aires by Britain's environment secretary, John Gummer, and Felix Cirio, Argentina's undersecretary of state for agriculture, livestock and fisheries. As part of the agreement, Britain will help Argentina to build up its expertise in biosafety, and the two countries will hold a joint workshop on the topic early next year.

A similar agreement is expected to be signed in the near future between Costa Rica and the government of the Netherlands. Britain and the Netherlands were the main countries responsible for drawing up the original draft of the guidelines, which some see as an alternative to a legally binding international treaty on biosafety; Gummer described the Argentine agreement as "an important step towards the safe application of biotechnology".

The move will put new pressure on those countries keen to see a formal international protocol, which signatories to the UN Convention on Biological Diversity, approved by the UN Earth Summit in Rio de Janeiro in 1992, have already agreed to explore.

A draft protocol has been drawn up by a panel of experts, in line with a decision passed at the first meeting of the convention's signatories held in the Bahamas last November (see *Nature* 372, 585; 1994). It was discussed at a UNEP biosafety meeting in Madrid in July, whose recommendations will now be put to the convention's signatory countries in Jakarta in November.

But although some UN members are enthusiastic about a legally binding protocol, others are more sceptical. The United States, Germany and Australia, for example, have all expressed strong reservations, concerned that signatories would be required to impose excessively rigid legislation on their domestic industry.

Members of a third group, which includes several European countries — in particular Britain and the Netherlands — say they support the idea of a protocol. But they also claim that the length of time required for such a document to be agreed, signed, ratified and subsequently implemented suggests that voluntary guidelines offer an alternative approach that should be pursued in parallel.

This was the spirit in which the guidelines were originally drawn up by Britain and the Netherlands in 1993. They cover a wide

spectrum of topics, ranging from agreement on the principal of risk assessment and risk management to the identification of bodies responsible for implementing safety regulations at both a regional and a national level.

The guidelines also specify mechanisms to be put in place to ensure that the transfer of genetically manipulated organisms between countries is accompanied by the appropriate information, a condition which officials say will provide useful guidance to researchers wishing to exchange material between laboratories in different countries.

Finally, in a move partly designed to increase the attraction of the guidelines to developing countries in which industrial nations seek to establish markets for their biotechnology products, the text specifies that the latter will help the former to develop "human resources, training and institutional capacities in order to achieve maximum benefit from biotechnology".

To the clear satisfaction of both British and Dutch officials, international discussion on the guidelines — whose implementation will remain voluntary on both sides, leaving it to the country concerned to decide whether and if so how its commitments will be enshrined in legislation — was taken over at the end of last year by UNEP, which says that they are entirely compatible with parallel efforts on a binding protocol.

The European officials now hope that Argentina and Costa Rica will be the first of many developing and newly industrializing countries that will agree to sign up. This will help such countries, they say, to attract foreign investment from pharmaceutical and other biotechnology companies.

Several countries in South-East Asia, for example, which have recently been making a substantial investment in their domestic biotechnology efforts, and are keen to open up markets for the results of such efforts in the West, are believed to be among those currently considering whether to do so.

Public opinion in Argentina has been sensitive to biotechnology safety ever since strong international criticism was voiced in the early 1980s of experiments with a genetically engineered vaccine against foot and mouth disease. The Argentine officials are therefore keen to display their social responsibility by agreeing to the UNEP guidelines.

UNEP has recently completed a series of regional meetings on the draft guidelines, and is preparing a final, global consultation at which supporters of the guidelines hope that international consensus — even given continuing claims from environmental groups that a voluntary approach to biosafety is insufficient — will be reached on both their value and content. **David Dickson**

Russian scientists threaten campaign of protests over funding

Moscow. Scientists from the Far Eastern branch of the Russian Academy of Science, which is based in Vladivostok, have threatened a campaign of civil disobedience in protest at the lack of funding from the central government.

In a letter addressed to President Boris Yeltsin at the end of last month, members of the trade union committee of the academy said that they were issuing a "last warning" to the leadership of the country about their plight.

"If political leaders do not pay attention to our problems and start observing the law on those parts of the budget that deal with the financing of science, we shall reserve the right to take the most extreme actions," wrote the scientists.

Committee members complained at a press conference in Vladivostok on 30 August that science in the Far Eastern region of Russia is in danger of disappearing completely due to a continuing shortage of funds, research materials and technical support.

As an example, committee members point out that marine biologists in the area are unable to carry out any research because a lack of funds means that the scientific fleet is not in operation.

Veniamin Myasnikov, deputy chairman of the Far Eastern branch of the academy, says one of the major problems is that being so far from other cultural centres, science in the region is relatively undeveloped, and most scientists have come from western or central parts of Russia.

As a result, unlike their colleagues in other cities, those who lose their job in a research institute or laboratory find it necessary to return home, as they cannot afford to take on temporary work while awaiting for new research funds to arrive.

Myasnikov claims that their departure only makes the situation worse for those who stay behind. "When the most qualified people, whom we had been pleased to welcome to Vladivostok many years ago, begin to abandon it, they leave behind an intellectual desert," he says.

The situation is not entirely bleak. According to Myasnikov, scientists at his institute have been able to win contracts from outside commercial bodies such as banks, factories and even local stock exchanges for setting up computerized control systems.

But Myasnikov admits scientists in other disciplines, such as geology, are less fortunate because their skills are in less demand from the private sector — one reason that geologists were the main organizers of last month's protest. **Carl Levitin**

Aid groups back challenge to neem patents

London and New Delhi. An international coalition of more than 200 aid and environmentalist groups is backing a request to the US Patent and Trademarks Office (PTO) to withdraw a patent issued to the multinational company W. R. Grace on a method for extracting an active insecticide from the Indian neem tree.

The groups, which include several Indian organizations representing small farmers, are claiming that the patent is invalid because native knowledge of the tree, *Azadirachta indica*, whose extracts have been used for centuries not only to control insects but also for a range of medical purposes, should be regarded as 'prior art'.

Their request to the PTO follows a similar move in Europe at the beginning of June, organized by the Greens in the European Parliament. This challenges a patent issued earlier this year by the European Patent Office (EPO) jointly to Grace and the US Department of Agriculture (USDA) on a technique for using a chemical extract from the neem as a fungicide.

Both moves, which extend a campaign launched in India two years ago, are intended to focus attention on the way that the patent system permits multinational companies to lay claim to techniques based on the indigenous knowledge of Third World countries, if they are able to extend this knowledge using modern scientific methods.

Grace and other multinationals claim

that their only responsibility is to demonstrate to patent authorities that the specific techniques for which they are applying for a patent meet conventional criteria for novelty and inventiveness (the PTO has already granted over 50 patents on neem-based products from toothpaste to contraceptives).

The US patent under challenge, for example, refers to a technique Grace claims to be unique for extracting the chemical azadirachtin from neem tree seeds in a way that allows the extract to be stored for far longer than when it is extracted by traditional techniques.

Those seeking a re-examination of the US patent point out that long-term storage is in little demand in India, since the extract has traditionally been used soon after its preparation. But they claim to have evidence of several small Indian companies which have been engaged in developing storable neem extract, and the outcome of their challenge may depend on whether the practices of these companies will stand up in court as 'prior art'.

A spokesman for Grace, however, says that the patent concerned does not concern the processing of the extract, but only the unique formulation of its active ingredient, azadirachtin, without which the natural active ingredient degrades quickly. The company says that the claims against the patent, which have been filed in Washington

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Neem: patent battle ranges from India to America.

by Jeremy Rifkin, director of the Foundation for Economic Trends, are "incorrect and without merit".

The neem issue is particularly sensitive because it has come to symbolize conflict between traditional and modern economic systems. Public protests against companies such as Grace delayed the passage through the Indian Parliament of new legislation on intellectual property designed to bring India's patent laws in line with those in the West as part of the outcome of the Uruguay round of the General Agreement on Tariffs and Trade.

Indian conservationists such as Vandana Shiva, director of the Research Foundation for Science, Technology and Natural Resources in Dehra Dun, one of the leading campaigners against the neem patent, hope that their campaign will draw world attention to the way that multinational corporations are patenting the biological resources of poorer nations.

"American people have only heard of software piracy," says Shiva. "What they don't know is that their companies are engaged in piracy of the worst kind in trying to profit from the traditional knowledge of India's farmers, who have been using neem as a pesticide for generations."

M. D. Nanjudaswami, head of the powerful Karnataka state farmers association, is confident that the patent will eventually be revoked. "If not, it would mean that indigenous populations around the world will not be able to freely use many of their local biological resources which they had developed and nurtured for centuries." Two years ago he led a widely publicized demonstration against a factory in Karnataka that supplies neem extracts to the US company.

One major concern to Indian farmers is the long term impact of the patent on the availability of neem seeds. With Grace already prepared to pay up to US\$300 per tonne of neem seeds, what used to be a free resource has now become a highly priced one. Shiva claims that a shortage of seeds will eventually force India's farmers to rely on Grace's product.

David Dickson and K. S. Jayaraman

Neem unsheaths contraceptive potential

New Delhi. While the pesticidal quality of neem (*azadirachta indica*) is the main reason for the tree's attraction to multinational companies, scientists at the Defence Institute of Physiology and Allied Sciences (DIPAS) in New Delhi have filed a claim on a substance that they have isolated from neem oil which kills sperm on contact.

The substance DK-1, which the scientists hope to use as a vaginal contraceptive in the form of a cream or pessary, has been isolated from the volatile fraction code-named NIM-76 separated from neem oil. According to M. Selvamurti, DIPAS director, the substance is a potent germicide at a concentration of less than 0.2 per cent.

Pharmacological and acute toxicity trials on rats, rabbits and guinea pigs, carried out at the Central Drug Research Institute in Lucknow, have cleared DK-1 for phase-one human trials, due to begin in November. Selvamurti says these will be followed by a large-scale trial in 1996.

The institute has transferred the technology to two Indian drug companies who will scale-up production of the neem con-

traceptive to produce the supplies needed during the phase-two trial and subsequent marketing. DIPAS scientists say that neem's historical role as part of Indian folklore may make it more socially acceptable as a contraceptive than conventional birth control pills.

Tests have shown that DK-1 is stable at 45°C. and has a shelf-life of at least six months. The neem isolate, says Selvamurti, has been subjected to the rigorous tests needed for pharmaceutical regulation. The contraceptive formulation will be licensed — unlike other herbal preparations, whose biological effects tend to show considerable variation.

Two more substances isolated from neem bitters also showed promise as potential contraceptives. But DIPAS intends to conduct trials only after it brings the first neem contraceptive onto the market. One of the substances, coded DNM-5, prevents implantation when administered orally in the early stages of pregnancy. The other fraction DNM-7 acts as an abortifacient. DIPAS has filed patent applications for all the three. **K. S. J.**

Teraflop computer hits a Republican nerve

Washington. The semiconductor company Intel, best known as the producer of microchips for personal computers, is to build what it describes as the world's most powerful computer under a \$50-million agreement announced last week by Hazel O'Leary, the US Secretary of Energy.

The machine will use about 9,000 of Intel's next-generation microprocessor, the P6, to deliver 1.8 teraflops — 1.8 million million floating-point operations per second — of computing power. It is to be built at the Sandia National Laboratory, which is run by Sandia on behalf of the Department of Energy, and should be operating by the end of next year on Sandia's main site at Albuquerque, New Mexico.

"It's exciting we can create a system ten times more powerful than the fastest supercomputer in the world today using the same chips we'll be putting into desktop PCs," says Andrew Grove, Intel's chief executive.

Although the computer is primarily intended for use by nuclear weapons designers to simulate nuclear explosions, Sandia officials say that it will be available for much of the time to other energy department scientists working on problems that require large amounts of computer power.

O'Leary made the announcement in Congress immediately before a joint hearing of two House of Representatives subcommittees into the future of the network of laboratories, which employ 59,000 people.

The timing appears to have been deliberately chosen to foster tensions between her Republican opponents in Congress, who are deeply split over whether to fund this kind of collaboration between the energy department laboratories and US industry.

Steven Schiff (Republican, New Mexico), the chair of the basic research subcommittee of the House science committee, whose district contains the Sandia laboratory, warmly welcomed the deal, and attacked unnamed Republican colleagues who have been dismissive of such projects.

"This is exactly the kind of partnership which some colleagues have criticized, many of them calling it corporate welfare," said Schiff. In his view, however, the agreement was "exactly what we need", and will benefit both US industry and the laboratories.

But Dana Rohrabacher (Republican, California), chair of the energy and environment subcommittee, which held last week's hearing jointly with Schiff's committee, is one of those who has branded technology agreements as corporate welfare, and sought to eliminate their funding (see *Nature* 376, 106; 1995).

The new teraflop computer is the first machine to be ordered under the energy department's new, ten-year Accelerated Strategic Computer Initiative (ASCI). Having decided that the computer would be built at Sandia, the department invited all the leading supercomputer suppliers —

including Cray and IBM — to bid for it.

But Intel was always considered to be a strong contender, as its parallel processing machines are already established at Sandia. A spokesperson for Cray, the world's leading supercomputer supplier, said it was "not



surprised at the outcome", but expected to fare better when contracts for future ASCI machines are placed at other laboratories.

European and Japanese computer suppliers have long complained that the US government uses direct subsidies to boost the global dominance of the US supercomputer industry, and this deal will do little to reassure them. All the money comes from the US nuclear weapons programme. But O'Leary left little doubt last week that a main objective is to reinforce US industrial dominance. "The US is known for its leadership in several technology areas," she said. "One of them is supercomputing. If we were not to aggressively 'push the envelope', the US would stand to lose its leadership."

House Republicans appeared to be in some disarray at the subsequent hearing about how best either to reform or reduce the Department of Energy laboratory system, lending comfort to those who are hoping the network can survive basically intact.

Bob Galvin, chairman of Motorola, and principal author of a highly critical report on the laboratories published earlier this year, called again for their "corporatization", a process under which a panel of trustees from industry and academia would operate each of the laboratories on a block grant from Congress.

But some committee staff say that Galvin's apparent belief in the innate superiority of businessmen over congressmen in taking strategic decisions did not go down well with committee members.

Even the conservative Rohrabacher ridiculed Galvin's vision, pointing out that if Congress were to entrust private businessmen with looking after public funds, they would soon be "taking money out of here by the wheelbarrow load". **Colin Macilwain**

X-rays, not radium, may have killed Curie

Paris. Marie Curie's final illness and death from the effects of radiation may have been due to her use of radiography during the First World War, and not, as generally believed, to exposure to radium, the element she discovered.

An opportunity to carry out an analysis of the radiation levels of her remains arose earlier this year, when Curie (right) became the first woman to be given France's highest honour, burial in the national mausoleum, the Panthéon (see *Nature* 374, 751; 1995).

Because of concern about the possibility of radioactivity escaping from the corpse, her exhumation was carried out under the control of the French Office de Protection contre les Rayonnements Ionisants (ORPI).

Curie's body was found to be enclosed in a wooden coffin, surrounded by a lead coffin, which itself was inside a further wooden coffin. ORPI found that the level of radiation caused by radium within the

interior coffin was, at 360 becquerels per cubic metre, significantly higher than the 13 Bq m⁻³ found at the entrance to the cemetery.

But the level was still well below the maximum accepted safe levels of public exposure to radium of 7,000 Bq m⁻³. Given that the half-life of radium is 1,620 years, ORPI has concluded that Curie could not have been exposed to lethal levels of radium while she was alive.

Although Curie's laboratory was highly contaminated with radium, an ORPI official points out that radium poses risks only if it is ingested either orally or through the skin.

ORPI therefore speculates that Curie's illness was more likely to have been due to her use of radiography during the First World War, when precautions to protect against X-rays had not yet been introduced. **D. B.**

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France shifts research focus on to new regional centres

Marseilles. A large group of senior French research officials and politicians descended on the Mediterranean port of Marseilles last week to inaugurate two research institutes. Such a gathering of high-level officials outside Paris is rare, and symbolizes the importance of regional centres for the pursuit of two key research policies in France — decentralizing research from Paris, and concentrating different research bodies' resources within joint research institutes.

The two instituts fédératifs de recherche (IFR), as they are known, are part of a plan to create research 'poles'. These are loose federations of laboratories that bring together scientists from local research centres, universities, hospitals and industry to create a critical mass in particular research projects at a single site.

For example, the first of the two new centres, the Institut Jean-Roche, will house three laboratories of the national biomedical research organization, INSERM, and three from the fundamental research organization, the Centre National de la Recherche Scientifique (CNRS), at the site of the Faculty of Medicine of the Hôpital Nord, in Marseilles. Many of the laboratories were previously operated as joint laboratories with the University of the Mediterranean — formerly known as the University of Aix/Marseille II — which is also a partner in the new centres.

Research at the centre, which employs 148 full-time staff, including 73 researchers, will focus on cellular interactions in endocrinal, nervous and epithelial tissue, particularly on developing clinical applications, for example, for AIDS and cancer. François Couraud, the director of INSERM's Laboratoire de Neurobiologie des Canaux Ioniques, will head the new centre.

The second centre, the Institut Fédéraliste de Recherche de Biologie du Développement (IBDM), brings together nine INSERM laboratories and ten CNRS laboratories, at a site in Luminy, south of Marseilles. Research at the centre, which has 66 researchers, will link fundamental research on developmental biology with clinical research on genetic diseases. The cost of the operation is estimated at FF22 million (US\$4.32 million).

One of France's few medical genetics services is based at the nearby Timone Hospital. Jean François Mattei, who heads the hospital's paediatrics and medical genetics department, is also a member of the National Assembly (UDF, Bouches-du-Rhône) and an influential voice in French bioethics debates.

IBDM has sufficient laboratory space to bring in several new research teams, and is

negotiating for the transfer of research groups from Paris. But Christo Gordis, the head of the centre, concedes that enticing researchers and their families away from Paris remains difficult, despite the promise of good research facilities, *bouillabaisse*, and a sunny Mediterranean climate. Attracting top scientists from elsewhere in Europe seems to have been less difficult. Gordis himself is a German national, while Christopher Henderson, head of the centre's Laboratoire du Développement et Pathologie du Motoneurone Spinal, is British.

Indeed, one of the main goals of creating such regional research 'poles' is to decentralize research away from Paris and stimulate regional development. The greater Paris area, known as Ile de France, accounts for less than a fifth of France's population and only a quarter of its student population; but it carries out more than half of the country's publicly-funded research.

In 1992, the government plans to transfer around 4,500 researchers from Paris to regional centres by the end of the decade (see *Nature* 356, 373; 1992). This change has been promoted through CNRS, for example by recruiting two researchers to regional centres for every one in the capital, and investing in new regional centres offering well-equipped laboratories.

The second goal of such regional centres is to improve coordination between the various research organizations, particularly in the life sciences. Earlier this year the science ministry itself took control of a new national strategy for life sciences research, made up of 14 strategic programmes administered by ministerial committees, with such a goal in mind (see *Nature* 374, 206; 1995).

But the new government formed after the election of Jacques Chirac as president of France in May seems likely to hand over control of the programme to the research organizations themselves, preferring to support initiatives such as the creation of IFRs.

Speaking at last week's inauguration, Elisabeth Dufourcq, the secretary of state for research, told the heads of the research organizations that "I don't want to substitute my central ministry for your roles". She added that the national life science strategy would in future be administered in "real concertation" with the research agencies.

Supporting this approach, Philippe Lazar, director general of INSERM, said that there is a need "to respect the different mission of the research organizations, but bring together their resources". The creation of IFRs, added Guy Aubert, the director-general of CNRS, will allow "increased firepower" with the same resource, by sharing the costs of equipment and overheads. **Declan Butler**

UK urged to seek role as 'mecca' for top graduates

Newcastle-upon-Tyne, UK. Sir Martin Rees, the astronomer royal, suggested this week that the British government should make a deliberate effort to turn Britain into "the country of choice" for top-ranking graduate students whose first instinct is to go to the United States.

Delivering his presidential address to the annual meeting of the British Association for the Advancement of Science (BA), he also called on the government to allow defence scientists greater freedom to discuss their work with colleagues in the rest of the scientific community.

Rees, who is Royal Society research professor of astronomy at the University of Cambridge, claimed that the quality and traditions of the best UK research institutions — as well as the primacy of English as the language of science — suggested that it should be possible for them to match the "blandishments" of the United States for internationally mobile talent.



Rees: UK could exploit its advantages further.

"Our universities have a fine record for attracting overseas students, but we should surely press it further," Rees told the BA meeting at the University of Newcastle. "Britain could surely exploit more fully its manifest comparative advantage as a magnet for talent, a location for research centres, and as an incubator for discovery and invention."

In his comments on government secrecy, he compared the situation in Britain to the relative openness of US defence research establishments. Claiming that pervasive secrecy inhibits well-informed and open debate, Rees said that he had had contact with many physicists from institutions such as the Los Alamos and Lawrence Livermore National Laboratories — but none with their British counterparts.

Rees also expressed concern about the dangers of imposing excessive *dirigisme* and short-term financial accountability on the research community. In particular, he warned that excessive efforts to impose a "business-like perception of efficiency" on the research community could backfire, in particular by discouraging the most creative individuals from pursuing a research career altogether. "We need to be businesslike," admitted Rees. "But that doesn't mean that we should operate too much like a business." David Dickson

Virus infection of baboons

SIR — Translation of baboon bone marrow is being considered for restoring the immune system of HIV patients¹. Concern has been expressed that such transplantation could result in the emergence of lethal new viruses in the human population. In this respect, simian haemorrhagic fever virus (SHFV) should be seriously considered as a threat.

SHFV is a member of an as-yet-unnamed new family of positive-stranded RNA viruses². SHFV is endemic in populations of three genera of African monkeys — baboons (*Papio anubis* and *P. cynocephalus*), patas monkeys (*Erythrocebus patas*) and African green monkeys (*Ceropithecus aethiops*). SHFV seems to establish persistent, perhaps lifelong infections in these monkeys without causing any clinical symptoms. However, artificial transmission of SHFV to Asian monkeys of another genus, namely rhesus monkeys (*Macacca mulatta*, *M. arctoides* and *M. fascicularis*), results in haemorrhagic fever that is generally fatal within 2 weeks after infection. Devastating epizootics among rhesus monkeys have occurred in monkey colonies in the former Soviet Union in 1967, the United States in 1967, 1972 and 1989 and in England between 1966 and 1969 with the loss of 1,000–2,000 monkeys. During the 1989 epizootic, which involved three monkey facilities in the United States, Ebola virus was also detected in some monkeys³ as discussed in the book *The Hot Zone*, by Richard Preston (Random House/Doubleday, 1994) but it has not been resolved to what extent the rhesus monkeys died from infection by SHFV or Ebola virus. This is a definitive example of a transmission of a virus that causes only asymptomatic infections in its natural primate hosts, including baboons, to primates of another genus with devastating consequences.

There is no indication that any humans became infected with SHFV during any of the SHFV epizootics. However, transmission of SHFV from African to Asian monkeys seems to occur only through accidental transfer of blood; initial transmission in epizootics is believed to have occurred by mechanical means through the use of unsterilized syringes. In contrast, transmission between rhesus monkeys is through the respiratory route and is very efficient. Unfortunately, very little information is available on the prevalence of SHFV in African monkey populations and detection of the virus is problematic. SHFV isolates generally grow poorly in cell cultures and infected monkeys often have only low levels of antiviral antibodies. The most sensitive method of detection is inoculation into rhesus monkeys, but this method is very expensive. It is

obvious that transplantation of humans with tissues from SHFV-infected baboons would result in transfer of considerable amounts of the virus and could result in selection of a variant that can replicate in humans.

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Benefits of placebos

SIR — Recent correspondence shows that placebo-controlled trials have their drawbacks. Beatrice Golomb¹ suggests that studies should be carried out to test for possible specific effects of placebo agents so as to eliminate an eventual confounding factor in the interpreting of double-blind study results. Justifiable as it seems from a scientific point of view, this does not appear to me a cost-effective approach. Recent developments show that the costs of new drugs that come to the market will not be automatically reimbursed by health assurance systems. In many countries, the market prices of drugs for one and the same indication are compared and drugs with the lowest daily costs are privileged. The need for extra studies to test for possible specific effects of placebos will increase drug development costs and thus market price. An alternative would be not to use placebos for the control of clinical studies, or to accept small effects of a placebo where its use cannot be circumvented. There may be good reasons to use no placebos for the control of clinical studies². Usually, however, two or more placebo-controlled (pivotal) studies are required to convince the medical profession and health authorities of the therapeutic value of a drug for a readily identifiable category of patients.

As Golomb rightly states, an apparent positive, negative or null effect of a drug may be the consequence of a negative, positive or same-direction effect of a placebo with which it is compared. Because it is not realistic to expect a pharmaceutical company to invest a huge amount of money in the development of a drug that is not viable, false negative effects are unlikely to occur and, if they occur, are taken for granted. Similarly, a lack of significant difference between active and placebo treatment is always a reason to abandon a project and not to invest more money just to confirm whether the placebo is as (in)effective as

the (in)active drug. In the worst case, a drug is credited for effects that it does not produce because by chance there had been negative effects of the placebo(s) used in the pivotal studies. Even then, this may not be harmful. It is generally assumed that when there is a moderate difference between two treatments in their effects on some specific disease outcome, this difference might be larger or smaller in other patients (studies), but it is unlikely to be reversed³. Besides, a compound with no specific effect (a placebo), but efficacious in the treatment of a disease when adequately administered and free of any toxicity, may be the best 'drug' that one can imagine.

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Science in Israel

SIR—Your supplements on science in different countries are to be commended, and that on science in Israel (*Nature* **376**, 717–732; 1995) is no exception. Israel harbours a promising potential in science in many various fields, from the computer sciences, through energy sources and water desalination to desertification, which has been augmented by the arrival of immigrant scientists from the former Soviet Union.

But there is a growing concern about the future of science and research and development (R&D) in Israel especially in basic science. This includes the state of science, fields of activities, centres of excellence and the shaping of science in the future, the cost of research, 'big science' and international cooperation, funding and the need for central (government) support both nationally and internationally; higher education, present and future, particularly with the call for higher education for all. Basic science is a major component of R&D in Israel, chiefly in versatility and output, although applied and industrial R&D surpass basic science in expenditure and manpower (scientists and engineers). Israel is first in the world in the number of scientific papers published per person.

Your supplement dwelt entirely upon applied and industrial-related R&D. I hope you will be able to write about basic science in Israel in some future issue.

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Big Bang not yet dead but in decline

The latest measurements of the Hubble constant make the Big Bang account of the origin of the Universe more dependent on the coincidence of numbers than it has so far been. But it remains the only theory in the field.

Is there a crisis in cosmology, or is it that the latest measurement of the Hubble constant is yet another of those numerical disagreements that plague the field from time to time? That is the question inevitably prompted by last week's article by N.R. Tanvir *et al.* (*Nature* 377, 27–31; 1995). Their chief conclusion, based on the accurately known recession speed of the Coma cluster of galaxies, is that the Hubble constant (otherwise H_0) implies an age of the Universe much smaller than the known ages of the stars in globular clusters in our Galaxy.

The obvious question is how that can be if the stars in the Galaxy were formed within the lifetime of the Universe. The obvious answer is that the result, the third of its kind in under a year, makes a nonsense of the standard Big Bang view of how the Universe began. But even those who are persuaded on other grounds that the Big Bang is a fairy story, perhaps for no better reason than that it is "too good to be true", had better conduct themselves with circumspection in the months ahead.

When the Hubble Space Telescope (HST) was built in the 1970s, it was generally understood that one of its chief purposes was to help construct a better distance-scale in the Universe. At that stage, it was also widely understood that the identification of Cepheid variables in more distant galaxies would play a crucial part in the process. (Cepheid variables are late-type stars whose period of oscillation is unambiguously related to their absolute luminosity.) Two of the three potentially disconcerting articles published in the past year have indeed been based on data gathered by the HST. All of them have turned on data gathered from Cepheids.

The three values of H_0 published in the past year are interesting in themselves. M.J. Pierce *et al.*, using the Keck telescope on Hawaii, reached the conclusion that H_0 is $87 \pm 7 \text{ km s}^{-1} \text{ Mpc}^{-1}$ (where 1 Mpc is a million parsecs or roughly three times as many light-years). That result hung on measurement of Cepheid variables in the galaxy NGC4571, one of the galaxies of the Virgo cluster (*Nature* 371, 385–389; 1994). A few weeks later, there appeared Wendy L. Freedman *et al.* (*Nature* 371, 757–761; 1994) with a value of $H_0 = 80 \pm 17 \text{ km s}^{-1} \text{ Mpc}^{-1}$, based on HST mea-

surements of Cepheids in another galaxy in the Virgo cluster, called M100. Now we have $69 \pm 8 \text{ km s}^{-1} \text{ Mpc}^{-1}$.

The temptation to regard either the simple average ($78.5 \text{ km s}^{-1} \text{ Mpc}^{-1}$) or some weighted average of these values as a better approximation to the "true" value should be resisted. All three groups of authors are careful to emphasize that their results entail chains of inference that may be false. The Virgo measurements suppose that the two galaxies concerned are near the core of the cluster, for which there is circumstantial evidence but no proof. Tanvir *et al.* rely on a different chain of inference: Cepheids in M96, a member of the Leo I galaxy cluster at a distance of about 12 Mpc, and the use of "consensus" estimates of relative distance to get from there to the Coma cluster, which is compact enough and far enough away ($105 \pm 12 \text{ Mpc}$) for the motion of individual galaxies to be irrelevant to the recession of the cluster as a whole. At this stage, it must be very hard to be sure that there is now some common false assumption in all three chains of inference.

How to resolve the difficulty? More data are the obvious solution. With HST in such good shape, there is no reason why it should not be used to look for Cepheids in almost all the galaxies of the Virgo cluster, for example, getting rid in the process of the continuing uncertainty about the structure of the Virgo cluster, which would be worthwhile in itself.

Those with their knife into the Big Bang should also reflect that if the value of H_0 eventually settles down near the values recently published, it will be necessary to come to an understanding of why so many other measurements have given values that are numerically roughly half as big. Those responsible are not, of course, slouches. Among others, they include Alan T. Sandage, who has for decades been poring over Palomar Mountain plates with the objective of defining a distance scale for the Universe.

It is also crucial that the apparent conflict between the recent measurements of H_0 and the age of the Universe depends on assumptions about the density of the Universe as well as on the particular solution of Einstein's equations used to describe the evolution of the Universe. The lower the density of matter in the

Universe, the more easily it is possible to reconcile a large value of H_0 with the existence of old stars. Freedman *et al.* put the point clearly last year; their value of H_0 is compatible with stars as old as 16 billion years only if the density of the Universe is a few per cent of the so-called "critical density" separating indefinite expansion from expansion followed by collapse.

That is what the density of the Universe seems in reality to be if its mass is represented by the visible galaxies and if there is no substantial amount of "missing mass". Many of those who are inherently suspicious of the Big Bang have been persuaded into that frame of mind by realizing that the search for the missing mass has much in common with the fruitless search for the lumeniferous aether at the turn of the present century; some of them may now suspend their disbelief.

That does not eliminate the need for some hidden source of mass to account for the way in which the outer parts of spiral galaxies (such as ours) rotate more quickly than can be explained by the visible mass concentrated in their inner regions. There is also the problem of what holds clusters of galaxies together gravitationally; the simplest assumption is that there is invisible mass in the shape of intergalactic gas (which is sometimes made visible by its X-ray emission when made very hot by collapse or by shock waves). Nobody may know what the missing mass consists of, but there can be no doubt of the reality of some of it.

In short, the new measurements of H_0 are not the death of the Big Bang, but merely a further sign of its fragility. There is also ample evidence on the other side, of course — the cosmic abundance of isotopes such as deuterium and ^3He for example. Naturally, it is not comforting that the truth on such a crucial question as the origin of the Universe should hang on numbers; qualitative arguments would be preferable. Perhaps the time has come for a reinvestigation of the origin of globular clusters, which contain the embarrassing oldest stars. The minute it is suggested that these structures are so exceptional that they must be survivors from an earlier stage in the evolution, the Big Bang will have given way to continuous creation. That will be a turn-up for the book.

John Maddox

Homeostasis without a GLUT

Mike Mueckler and Geoff Holman

THE blood glucose concentration in mammals is maintained at a constant level by an intricate interplay between circulating hormones and a multitude of cellular proteins involved in its production and disposal. This homeostatic mechanism can deal with most of the fluctuations in glucose supply associated with starvation and the dietary excesses of the overindulgent. It has defeated the most elaborate endeavours of experimental scientists to perturb it. Now Katz *et al.* show on page 151 of this issue¹ that it copes even in mice lacking GLUT4 glucose transporters in their fat and muscle. These knockout mice exhibit only small changes in their steady-state blood glucose levels and their ability to dispose of an oral glucose load; so they are not diabetic, although they appear to be moderately insulin-resistant.

Four facilitated-diffusion-type transporters in the plasma membrane are responsible for catalysing the flux of glucose into and out of the blood circulation (see figure). GLUT1 (ref. 2) in the blood-brain barrier and GLUT3 (ref. 3) in neuronal cells supply the high and constant levels of glucose needed by the mammalian brain. GLUT2 (ref. 4) and GLUT4 (refs 5, 6) have distinct but complementary roles in maintaining a constant blood glucose level. GLUT2-catalysed glucose transport can increase blood glucose levels by supplying glucose from enterocytes and from liver. In addition, when circulating glucose levels are high, GLUT2 catalyses its downhill flux into liver and pancreatic β -cells.

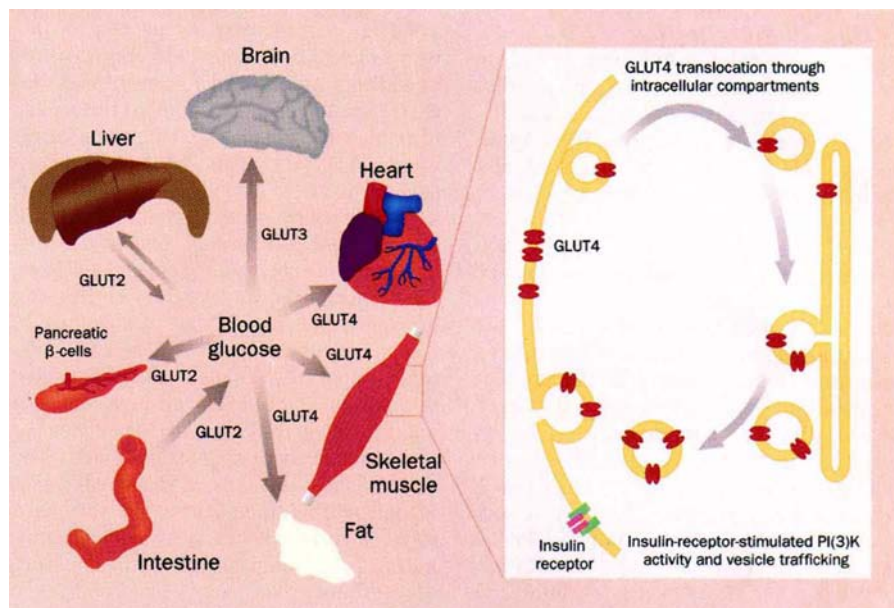
GLUT4 is of particular importance because it catalyses the rate-limiting step for glucose uptake and metabolism in skeletal muscle, the main site of disposal of blood glucose. When circulating insulin levels are low, as in the post-absorptive state, most of the GLUT4 resides in intracellular membrane compartments, where it is prevented from contributing to cellular glucose transport. After a carbohydrate meal, increased blood glucose levels stimulate release of insulin from the pancreas. The released insulin interacts with its receptor on fat and muscle cells and, in a mechanism that involves an interplay between signalling and membrane-vesicle-trafficking proteins, stimulates the redistribution of GLUT4 to the plasma membrane⁷. This results in increased cellular glucose uptake and metabolism and acts to lower blood glucose. This process of insulin-stimulated glucose uptake is defective in the muscle of patients with type II diabetes⁸ and so there is considerable interest in the mechanism of GLUT4 activation and the establishment of methods by which this

'insulin-resistance' can be overcome.

Studies in which GLUT4 is overexpressed in the skeletal muscle of transgenic mice demonstrate that transport is rate-limiting for muscle glucose disposal and that the level of muscle GLUT4 also affects the maximal rate of whole-body glucose disposal^{9,10}. Strikingly, diabetes could be prevented in homozygous diabetic (*db/db*) mice, which are genetically predisposed to obesity and insulin resistance,

The marked reduction in plasma lactate levels in the knockout mice suggests that there is a decreased flux of glucose through the muscle glycolytic pathway, which is responsible for the entry of the bulk of circulating lactate into the blood. If muscle uptake of glucose is limited, it may use circulating fatty acids as an additional energy source. But are the decreased fat stores observed in the GLUT4-knockout mice a consequence of high fatty acid utilization or of the inability to transport sufficient glucose for fat deposition in adipose tissue?

If indeed skeletal muscle glucose uptake is low, these animals might main-



The four plasma-membrane glucose-transporter isoforms (GLUTs) found in mammals have a well characterized tissue distribution. A fifth isoform is now known to be a fructose transporter. GLUT1 is present in most cell types but is very abundant in erythrocytes and the blood-tissue barriers of the brain and placenta. Sodium-linked glucose transporters enhance the flux of glucose from the intestinal lumen into enterocytes. GLUT2 is present at the basolateral surface of enterocytes, enhancing the flux from the enterocyte to the blood circulation. GLUT2 is also abundant in the liver and pancreatic β -cells. GLUT3 is most abundant in the brain. GLUT4 is only present in the insulin-target tissues of adipose tissue, skeletal and heart muscle. When circulating insulin levels are low, GLUT4 is associated with intracellular membrane compartments and vesicles. Insulin signalling promotes the translocation of GLUT4 to the plasma membrane where it enhances glucose uptake from the blood circulation for storage in cellular fat or glycogen deposits.

by overexpression of the human GLUT4 gene in fat and muscle¹¹.

The results of Katz *et al.*¹ might be considered surprising in the light of these earlier studies because a severe defect in glucose homeostasis is not evident in the GLUT4-knockout mice. How can one account for the almost normal blood glucose levels in these animals if skeletal muscle is the primary site of glucose disposal after an oral glucose load? Mice lacking GLUT4 must have somehow compensated for this deficiency.

Unfortunately, Katz *et al.* did not measure the glucose uptake by skeletal muscle of GLUT4-knockout mice, but there is indirect evidence for a reduction in glucose utilization in their skeletal muscle.

tain almost normal blood glucose levels because of feeble hepatic glucose regeneration from the low levels of circulating lactate. Insulin action on liver may suppress this glucose production but increase the utilization of glucose moving into this organ through GLUT2: these interactions would be consistent with the low lactate and increased insulin found in the blood of GLUT4-knockout mice.

It is possible that pathological changes may have occurred in the knockout animals which counteract any increase in blood glucose. For example, a decrease in the amount of glucose absorbed in the gastrointestinal tract after feeding glucose may mask a defect in the ability of skeletal muscle to handle a glucose load. Adminis-

tration of glucose by an intraperitoneal or intravascular route may thus uncover more striking abnormalities in glucose tolerance in these animals. In addition, glucose may be lost in the urine as a result of renal malfunction. Functional alterations in these or other organ systems may be associated with adaptations to the altered metabolic milieu or to pathological changes resulting from altered blood flow

due to the cardiac hypertrophy described. Direct evaluation of glucose utilization by individual tissues *in vivo* and *in vitro*, evaluation of food intake, and measurement of urinary glucose levels and renal haemodynamics will undoubtedly answer some of these questions.

Further characterization of these mice may yield interesting data on their ability to compensate for metabolic defects during development. Indeed, as suggested by Katz *et al.*, further investigation of the compensatory mechanism by which these animals cope without GLUT4 may provide insight into mechanisms that can be exploited in the treatment of human type II diabetes. □

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POLYMER SCIENCE

Gourmet macromolecules

Philip Ball

THE difference between synthesis in small-molecule organic chemistry and in polymer chemistry has traditionally been much like the difference between dinner at the Ritz and a school meal. One is exquisitely and individually prepared step by step; the other is mass-produced by throwing the ingredients into a single pot and boiling for an hour. Lest polymer chemists be offended by this comparison, it should be pointed out that of the two reasons for the gastronomical differences, only one applies to polymer synthesis: it is not that the culinary skills of polymer chemists are any the less, but that their clients are less discerning. What matters, if one is producing a polypropylene shopping bag, is not that each molecule has an identical size and structure, but that the crude bulk determinants of the mixture, such as viscosity and molecular-weight distribution, be reproducible.

Several symposia at a recent meeting of chemists*, however, underscored the fact that polymer chemists can now serve up concoctions scarcely less impeccable than those of natural-product chemists. New synthetic strategies are providing opportunities to dictate the architecture of macromolecules to an extent that their mass spectra show the sharp single peak of an almost unimolecular sample rather than the gaussians and skewed-gaussians of old.

Dendrimers — macromolecules whose many-branched tendrils radiate out from a central core to create a globular morphology — have been much acclaimed already (see, for example, H. W. Gibson, *Nature* **371**, 106–107; 1994; D. A. Tomalia and P. R. Dvornic, *Nature* **372**, 617; 1995). New applications of these molecules continue to suggest themselves — they might, for instance, act as nanoscale single-molecule probe tips for near-field fluorescence microscopes (R. Kopelman, Univ. Michigan).

But great things are also predicted for the highly nonlinear hyperbranched polymers formed by the condensation of branched monomers of the type AB_x (J. Fréchet, Cornell Univ.; C. Hawker, IBM Almaden). Predicted by Paul Flory in the 1950s, the first example of these macromolecules was reported in 1988. In terms of macromolecular organization they occupy a middle ground between the precisely controlled dendrimers and the more or less uncontrolled conventional linear polymers. Because in general not all of the x B groups on every monomer will be condensed with an A group of another monomer, the residue of reactive B groups makes hyperbranched polymers of possible value as functional polymers (for example, as adhesives); but whereas dendrimers display their reactive groups solely at the surface of the macromolecules, in hyperbranched polymers these groups may be buried within the globular architecture, leaving

the polymer stable but with the potential to be thermally activated into a reactive form.

The earliest hyperbranched polymers contained threefold branch junctions based on AB_2 monomers, but very high degrees of branching can now be achieved by using controlled stepwise ('living') polymerization to make oligomeric 'macromonomers' with x ranging from 4 to 8 (Fréchet). Living polymerization methods, which have evolved largely over the past two decades, are surely amongst the most valuable tools to have entered the polymer chemist's kitchen. The chains of living polymers grow strictly by sequential addition of monomers to a reactive chain end, which is stabilized so as to prevent the chain termination or chain transfer processes that, in traditional free-radical or condensation polymerizations, result in broad molecular-weight distributions and a mixture of products.

In an ideal living polymerization reaction, monomers are added to each of the initiator molecules at an equal rate, so that when the supply of monomer is exhausted all of the chains have more or less equal length, and the reactive chain ends remain 'living' in the sense that polymerization will resume if more monomer is added. Most living polymerization reactions have involved anionic or cationic reactive ends, stabilized by the presence of a counterion; but free-radical chain ends, stabilized by a free-radical capping agent that will bind reversibly without initiating new chains, can now also be used (Hawker) to provide a variety of controlled macromolecular architectures, such as star polymers and graft copolymers in which the star arms or grafted side chains are of equal length.

Precise control of polymer architecture is the hallmark of biology, which uses templating methods to realize this goal. The same specificity for an arbitrary but predefined monomer sequence has been accessible for some time now through the solid-state methods used to construct stretches of polypeptides or nucleic acids with particular sequences, an approach that can, in principle, be used to make chains of any length but which in practice becomes very time-consuming for chains longer than 30 or so monomers. But the speed with which, say, the 4^N possible sequences of DNA N -mers can be generated may be greatly enhanced by using photolithographic techniques to build up pixelated combinatorial arrays (S. Fodor, Affymetrix, California). By tethering these oligomers away from the substrate on polyethylene oxide strings, the arrays can be used for rapid and easy recognition of complementary sequences in free strands of DNA, fluorescently tagged so that the appropriate element of

*National Meeting of the American Chemical Society, Chicago, 20–24 August 1995.

the array 'lights up' when binding occurs. The primary use of these oligonucleotide arrays, now marketed commercially as biosensor-style chips, may be as a convenient way to write down sequence information in a form that would allow easy comparison of one genome against another.

Co-opting natural polymerization machinery to express purely synthetic genes is now possible (D. Tirrell, J. Beecher, Univ. Massachusetts at Amherst, see *Nature* 367, 323; 1994) by using recombinant DNA technology to incorporate artificial sequences, prepared by solid-state methods, into *Escherichia coli* plasmids. The fruits of this new approach are now starting to fall: for example, hybrid enzymes that are expressed with a ready-made synthetic polypeptide region that confers a useful materials property, such as self-

organization into films or electrochemical activity.

And finally, controlling the microscale morphology of polymers, rather than their macromolecular structure, can provide new microsculpted materials: examples range from patterned polymer sheets formed by letting capillary forces draw the monomers into a micropatterned mould (G. Whitesides, Harvard Univ.; see *Nature* 376, 581; 1995) to macroporous functionalized polymer monoliths for use in chromatography and bioreactors (Fréchet) to crosslinked, hollow microspheres of haemoglobin (M. Wong, Univ. Illinois). The latter, formed by ultrasonication of a solution of haemoglobin, could act as oxygen carriers, with similar sizes and flow characteristics to erythrocytes, in artificial blood. □

Philip Ball is an associate editor of *Nature*.

BROWN DWARFS

A bright future for faint stars

Lorne Nelson

WHAT distinguishes a star from a planet? Could we call Jupiter a failed star? In an attempt to answer these questions, astronomers have postulated the existence of a whole new class of astronomical objects called brown dwarfs^{1,2}, which, in essence, bridge the gap between stars and giant planets. On page 129 of this issue, Rebolo, Zapatero Osorio and Martín³ present compelling evidence that they have discovered one of these elusive objects in the Pleiades star cluster. Not only does their discovery confirm the predictions of astronomers that brown dwarfs must exist, but it also opens the door to studying a class of objects that may well constitute a significant fraction

of the baryonic dark matter that is thought to exist on all distance scales throughout the Universe.

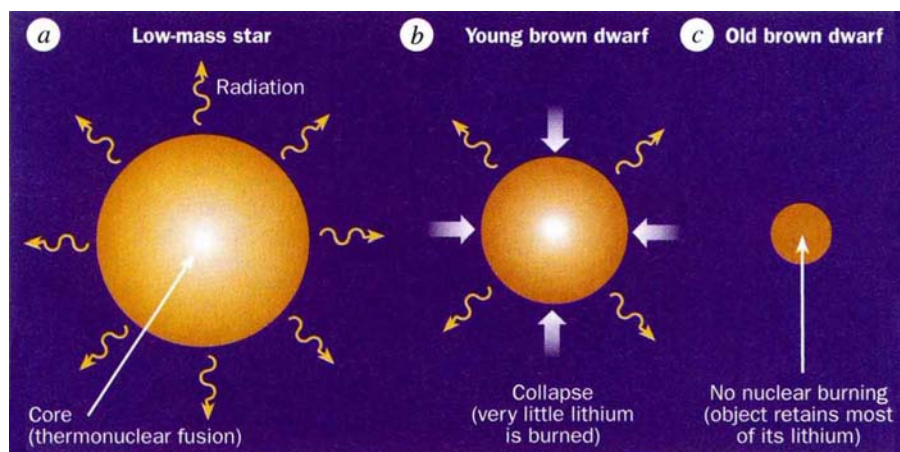
To understand how brown dwarfs differ from stars and planets, consider the Sun and Jupiter. Both were formed at the same time and are self-gravitating 'gas' spheres composed primarily of hydrogen. The Sun has a mass nearly 1,000 times greater than that of Jupiter and thus it has a very different internal structure. But suppose that we decreased the Sun's mass by a factor of ten and increased Jupiter's mass by the same factor. Would we still refer to Jupiter as a planet and to the Sun as a star? What if the factor were thirty?

Clearly, the mass of an object and the

process by which it formed are two of the important factors that must be considered when determining an object's classification (see figure). Stars are formed as a result of the collapse of a fragment of an interstellar gas cloud. As the star contracts, its internal temperature increases until it is able to sustain thermonuclear fusion. However, when the mass of the 'protostar' is less than ~ 0.08 solar masses, its internal temperature never becomes high enough to achieve stable hydrogen burning. Consequently, it continues to collapse (and cool) until electron degeneracy pressure halts further contraction. These objects are known as brown dwarfs and are often referred to as 'failed stars'. Giant planets also contract and cool during their evolution, but they are distinct from brown dwarfs in that they form by the cold accretion of matter in a gaseous disk rather than through the fragmentation of a gas cloud.

As a brown dwarf cools, it becomes increasingly faint. A 0.06-solar-mass brown dwarf whose age is equivalent to that of the Sun is about 100,000 times less luminous. Although this intrinsic faintness makes them extremely difficult to detect, advances in telescope technology during the past decade have spurred rapid progress. Astronomers are using a variety of techniques to search for brown dwarfs⁴, but looking for them in young clusters of stars that are relatively close to the Sun seems to be the most promising approach. Not only are young brown dwarfs much more luminous, but as the ages of clusters are reasonably well determined, the masses of brown dwarf candidates can be inferred and the validity of theoretical models can be examined.

Rebolo and colleagues discovered the apparent brown dwarf (Teide 1) in the Pleiades cluster. As the cluster is located about 400 light years away and is only about 100 million years old, it is an ideal location to search for brown dwarfs⁵. From its observed brightness and inferred surface temperature, Rebolo and colleagues conclude that Teide 1 probably has a mass of ~ 0.02 solar masses. Unlike previous claims for the existence of objects very close to the substellar limit, Teide 1 seems to have a mass that places it well below this limit. However, there appears to be a genuine discrepancy between the theoretical models and the observationally inferred properties of Teide 1. In particular, the inferred surface temperature of Teide 1 seems to be too low. The presence of molecules and possibly even grains in the atmospheres of brown dwarfs makes it difficult to calibrate the surface temperatures. At present, we cannot rule out the possibility that the mass of Teide 1 is as large as 0.07 solar masses, although even this is below the limit of 0.08 solar masses. This issue will probably be resolved when a new



Schematic comparison of stars and brown dwarfs. In the low-mass star (a), sustained nuclear burning of hydrogen in the core prevents the star from collapsing under its own gravity. In contrast, as a young brown dwarf (b) contracts, its internal temperature rises but never gets high enough to sustain nuclear fusion. In an old brown dwarf (c), the collapse is halted by electron degeneracy pressure. The object cools continuously and becomes very faint.

generation of synthetic (theoretical) atmospheres becomes available.

The Pleiades have also proved to be a fruitful hunting ground for another group of astronomers who very recently confirmed the existence of another brown dwarf using the 10-metre Keck Telescope⁶. This object (PPI 15) was discovered about two years ago by Stauffer *et al.*⁷, who suggested that it might be a brown dwarf on the basis of its low luminosity and spectral characteristics. Basri and colleagues⁶ were able to conclude that PPI 15 was indeed a brown dwarf after detecting lithium in its atmosphere. Unlike very-low-mass stars, brown dwarfs never become hot enough to burn their primordial lithium^{8,9}. Thus the authenticity of a potential brown dwarf can be checked by looking for the signature of lithium (and beryllium) in its spectrum. Clearly, this test must be carried out on Teide 1 — not a trivial task, as it will require many hours of telescope time on the Keck.

Because brown dwarfs are so difficult to detect, they are natural dark-matter candidates. Analyses of the number density of very-low-mass stars, appropriately extrapolated, indicate that brown dwarfs should be extremely numerous in the disk and halo of our Galaxy (and in other galaxies). Even though their number density is likely to be relatively large, their mass is so low that they need not make up a large fraction of the 'missing mass'. As Rebolo and his colleagues point out, the recent brown dwarf discoveries in the Pleiades are consistent with the hypothesis that brown dwarfs make up only a very small percentage of the mass of the cluster.

Regardless of whether nature has managed to hide substantial amounts of dark matter in the form of substellar objects, it seems that we are now in a unique position to test our theoretical models of stellar atmospheres and interiors. Perhaps we have finally passed a threshold in technology that will now enable us to detect large numbers of brown dwarfs. With the advent of new telescopes, such as the Space Infrared Telescope Facility, the future of brown dwarf astronomy looks brighter than ever. □

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Hedgehog keeps to the left

Lewis Wolpert and Nigel A. Brown

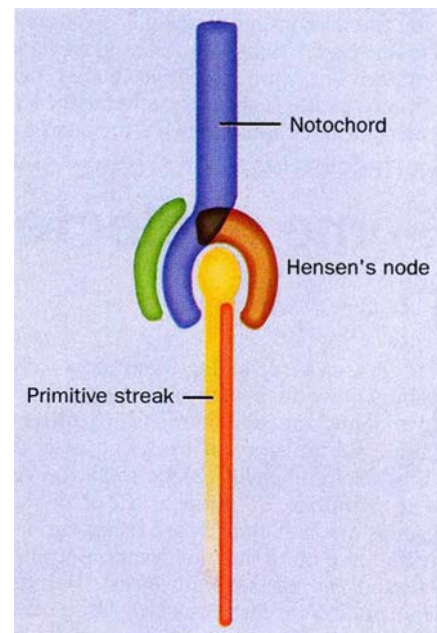
IN the evolution of developmental mechanisms, nature, it seems, has been rather conservative if not downright lazy; once a set of suitable signalling molecules had been discovered, little effort was made to find new ones. Levin *et al.*¹ have now shown that the embryo uses the same small set of molecules in the development of left-right asymmetry that is involved in patterning structures as superficially different as vertebrate limbs, insect wings and the spinal cord. This group contains members of the TGF- β family, which includes activin, and the omnipresent *hedgehog* genes.

For most of us the heart is on the left side, there are more lobes of the right lung than the left and indeed most internal organs are asymmetric. Although the few rare individuals with complete reversal of body asymmetry do very well, partial reversal can have serious consequences. Left-right asymmetry is consistent across all vertebrates and provides a lovely problem for developmental biologists. How does the embryo reliably distinguish left from right and then generate left-right structural differences? Left and right have no meaning on their own; they can only be defined once the anteroposterior and dorsoventral axes have been specified. It is for this reason that our left and right are reversed when we look in the mirror: our dorsoventral axis has been reversed.

The general expectation has been that answers would come from genetics because mutations have been described that can randomize left-right asymmetry or can completely reverse asymmetry². Cloning of these genes has been awaited with increasing interest. But the new excitement comes from a different approach. Levin *et al.* examined the expression of a number of patterning genes in the chick embryo at the time of gastrulation. At this stage the embryo is essentially a flat sheet characterized by a groove — the primitive streak — running along the anteroposterior axis, which has at its anterior end Hensen's node, the chick equivalent of the Spemann organizer in amphibians. The chick node and primitive streak have subtle but long-known left-right asymmetries³. Genes such as *goose-coid* and *FGF4* are expressed symmetrically in the node, and so too are genes expressed outside the node such as the *activin receptor IIb*, *Hoxb-8* and *engrailed*. By contrast, Levin *et al.* have shown that the *activin receptor IIa* is initially expressed more strongly on the right side of the streak and then in the right half of the node, whereas *Sonic hedgehog* becomes restricted to the left side of the node. At a later stage, *cNR-1*, a member of the

TGF- β family related to the mouse gene *nodal*, is expressed only to the left of the node region (see figure).

Can this pattern of expression be altered and could it affect asymmetry? Beads soaked in activin were placed on the left side of Hensen's node and caused the appearance of *activin receptor IIa* and the disappearance of *Sonic hedgehog*. Such embryos were thus no longer asym-



A composite of asymmetric gene expression domains in the chick embryo at stages 3+ to 7. Levin *et al.*¹ suggest that an activin-like molecule expressed on the right induces the *activin receptor IIa*, which represses *Sonic hedgehog* expression in the right of the node. Later, *Sonic hedgehog* induces *cNR-1* to the left of the node. Red, *activin receptor IIa* at stage 3+; brown, *activin receptor IIa* at stage 5; blue, *Sonic hedgehog* at stage 5; green, *cNR-1* at stage 7.

metrical with respect to *Sonic hedgehog* expression and when allowed to develop, asymmetry of heart looping was random, half being to the right and half to the left. *Sonic hedgehog* expression could also be made symmetrical with respect to the node by implanting, this time on the right side, a pellet of cells transfected with a virus so that they express *Sonic hedgehog*. This again caused randomization of heart looping.

A good case can thus be made for *Sonic hedgehog* playing a key role in specifying left-right asymmetry. When the expression of *Sonic hedgehog* is symmetrical then asymmetry is random, and similar to that in mice homozygous for the *iv* mutation. A crucial experiment to demonstrate a direct role of *Sonic hedgehog* would be to

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place an activin bead on the left and a pellet of cells expressing *Sonic hedgehog* on the right, thus causing an inversion of *Sonic hedgehog* expression. The expectation is that if *Sonic hedgehog* does control asymmetry then such embryos would all develop with reversed asymmetry.

These experiments open up a quite new way of investigating the mechanism whereby asymmetry is generated. The earliest molecular evidence for asymmetry is the expression of the *activin receptor IIa* on the right. Levin *et al.* suggest that this may reflect an earlier asymmetric expression of an activin-like molecule. But this still leaves open the question of why the activin-like molecule is only expressed on the right. However, as we have pointed out, any mechanism that can initially create a difference between left and right sides could provide the basis for

biasing a mechanism for the random generation of asymmetry⁴. It is the early specification of left versus right that is the true puzzle. We have suggested that this could involve a conversion of molecular asymmetry to the cellular level. In such terms *Sonic hedgehog* would be quite late in the cascade and reflect an interpretation process. In the search for initial asymmetries, one should perhaps hunt for a molecule like Vg1, which belongs to the same family as activin and which becomes localized at the vegetal pole in amphibia and is involved in axis specification⁵. It is possible, but no more than speculation, that a mechanism exists for localizing such a molecule asymmetrically.

In spite of these exciting new results a small dampener must be introduced; so far there appears to be no evidence for similar asymmetrical expression of these

genes in mammals or zebrafish. Moreover, mice in which the *activin receptor IIa* is knocked out have normal asymmetry⁶. For those with a leaning towards universal mechanisms, this is merely a temporary discouragement because we can rely on evolution's laziness. □

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VERTEBRATE PALAEOLOGY

Some neglected relatives

Robert Presley

MOLECULAR technology can now contribute powerful evidence on the probabilities of phylogenetic relationships, but the details of the story of evolution of body form depend heavily on the fossil record. The affinities of extinct, as of living, groups are best established using the distribution among them of synapomorphies (shared evolutionary advances), but the information on these can only be as complete as the fossil record. Two papers on pages 141 and 144 of this issue, one on the auditory apparatus¹ and the other on the forelimb², give new detail for an extinct subclass of mammals, and show how sensitive the interpretation of that record is to the chance preservation of detail.

Some extinct mammals, such as sabretooth cats and three-toed horses, are relatively famous in general knowledge. Not so the multituberculates, a subclass familiar only to specialists. This is ironic, because the span of their existence may well have been longer than that of any more famous group of mammals. The name 'multituberculate' derives from the molar teeth of typical species. These have longitudinal rows of uniform cusps used with an anteroposterior power stroke in chewing³. The earliest undisputed multituberculate family (Paulchoffatidae) is Late Jurassic but, surviving the Cretaceous extinctions, the group disappears at the end of the Eocene. Isolated molar teeth from the Rhaetic (Late Triassic), assigned to the haramiyid family, also show evidence of the unusual longitudinal chewing stroke, and past workers placed them within Multituberculata. Although recent work⁴ takes the more cautious line of handling haramiyids and multitubercu-

lates as sister groups, we are effectively looking at an extinct type of mammal which endured for 150 million years.

Multituberculate–mammalian kinship too has long been established by numerous cranial and body skeletal features⁵. The exact relationship to the three extant sub-Classes (monotremes, marsupials and placentals) is discussed in the new papers^{1,2}. Did they arise independently of



The Late Cretaceous multituberculate *Nemegetbaatar*, reconstructed with a somewhat sprawling stance (from ref. 7, with permission).

the others, or are they close to monotremes, but removed somewhat from marsupials-plus-placentals (therians)? Or do they all show enough concordant advances in the detailed anatomy of different systems to make independent evolution seem less probable?

Having the mandible as the sole lower jaw bone is a unique mammalian character. The small bones that develop behind, sutured into the lower jaw of other vertebrates, detach in mammalian embryos to

become specialized bones in the middle ear. It has long been known that the multituberculate mandible lacks any sutures for these more posterior elements in geological epochs when they still suture in all other Mesozoic mammals. This could imply that detachment of these small bones occurred independently in the multituberculates, to follow a different evolutionary path.

Malleus, incus and stapes, though rarely perfect, have been known since 1986⁶ in the large Eocene Chinese species *Lambdopsalis bulla* (named for its extraordinarily inflated auditory vestibule). Meng and Wyss¹ now describe fragments of the ectotympanic and the anterior process of the malleus. These show a number of features very similar to those in extant monotremes, providing direct evidence that the multituberculates had the whole array of mandible and middle ear elements, just as in extant mammals. Five bones are involved: ectotympanic, prearticular, malleus, incus and stapes. The hypothesis that this diagnostic middle ear complex evolved twice seems unlikely in view of the detailed similarities of association and anatomy of these elements. On this line of argument, monotremes and multituberculates may be close relatives, both are close to therians, and all are (monophyletically) mammals.

Recent work on the post-cranial skeleton has produced conflicting conclusions. A biomechanical study of a large number of bones from six Late Cretaceous species⁷ concluded that the multituberculate forelimb girdle is significantly more primitive than the therian. The picture, with strong support from the hindlimb girdle and axial bones, and consideration of well preserved markings of muscle attach-

MULTIPLE SCLEROSIS

More genes versus environment

Byron H. Waksman

ments, is consistent with an early mammalian radiation always retaining something of the ancestral sprawling gait. Two complete humeri from the Eocene have been published as stereophotographs and show features to support this⁸, including a marked residuum of the primitive torsion between humeral head and elbow condyle. Sereno and McKenna² now argue from the features of their beautiful specimen of a small, Late Cretaceous multituberculata forelimb that it was very like that of therians and was swung parasagittally over a considerable range. A corollary is that therians and multituberculata share this advance, not shown by monotremes. Non-palaeontologists should note that the methods used in these studies are not speculative descriptions of how the dead bones may have articulated and moved: a considerable number of osteological features are scored objectively to evaluate functional and phylogenetic correlations. However, many fossil specimens show cracks and distortions which can make biometrics inexact.

There is need for caution in relation to these new conclusions. The detail is obtained from one or two species in a long-lived radiation that must have adapted to a variety of niches. How likely is it that all multituberculata over this huge time span were restricted to low-frequency hearing? If the forelimb was advanced in a Cretaceous form and primitive in an Eocene form, from the same taeniolabidoid group, how secure is any assumption about the timing of the advance? We derive from marsupials and placentals knowledge of a broad range of adaptations, but the monotremes, platypus and echidna, are very specialized in locomotion and hearing, and we still only guess at the primitive ancestral form. With important new material coming from the efforts of Australian palaeontologists and from the recent American-Mongolian expeditions, we can hope that more well preserved skeletal finds will provide both interesting answers and surprises within this somewhat neglected part of our family history. □

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ON page 150 of this issue¹, Ebers *et al.* describe the results of a painstaking study of 15,000 patients suffering from multiple sclerosis (MS). Their conclusion is that the aggregation of MS cases within families is determined entirely by genetic factors and is not influenced by shared environmental factors like infection. This vexing genes-versus-environment issue has been convincingly resolved as a result of the authors' comprehensive screening of adoptive first-degree 'relatives' of a large, fully ascertained and well studied population of MS patients from fourteen clinics across Canada.

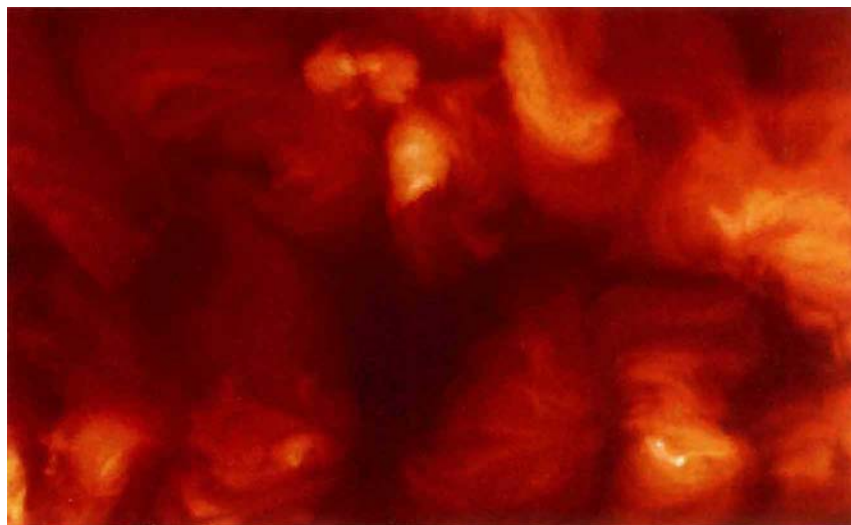
In a sense, these findings are not surprising. It is now widely thought that MS is the result of an immunologically mediated, quite possibly autoimmune, process. Susceptibility to MS seems to be determined, in part, by the genes that encode elements of the trimolecular complex involved in recognition by T cells. These

include the major histocompatibility complex (MHC), certain T-cell receptor peptide sequence polymorphisms and encephalitogenic peptides of brain protein antigens; also implicated are genes governing elements of the immunologically determined inflammatory process. The high concordance of MS in monozygotic twin pairs and in first-degree relatives of individuals with MS, cited by Ebers *et al.*¹, is an expression of these genetic influences.

But although Ebers and co-workers were unable to detect any effect attributable to shared environment, things may not be quite so simple. These authors only addressed the question of MS prevalence among non-biological 'relatives' living with an MS case, most of whom do not carry genes for MS susceptibility. But shared environment undoubtedly does play a part in the induction of disease among biological relatives who live with

SOLAR PHYSICS

Coils of flame



J. Klimchuk/Yohkoh

THESE fiery coils would have been the delight of any Renaissance painter, could he only have seen them. But the image shown here is not a snippet from the head of some Titian beauty. Instead, the arching, twisted loops are an X-ray telescope's view of the solar corona, where the gas — fiery indeed — is at a temperature of several million kelvin. How the gas is heated to this extent was the question exercising James Klimchuk and Lisa Porter, whose research is reported on page 131 of this issue and in a paper to appear in the *Astrophysical Journal*. From observations of long-lived coronal loops made with the soft X-ray telescope aboard the Japanese Yohkoh

satellite (the name means 'sunlight'), they have determined the temperatures and densities of individual loops, and from this derived a scaling law for loop length with gas pressure. The various theories for the mechanism of coronal heating make different predictions for what this scaling law should be, so — an unusual feat in this area of research — Klimchuk and Porter have supplied an observational means of discriminating between rival theories. To untangle the tale further will, they suggest, best be done by making direct measurements of the magnetic field strength in the loops.

Lindsay Nightingale

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the index case, and who are susceptible to MS.

Data obtained in an earlier study by Ebers' group, which looked at sibpairs in MS families, supported a role for genes and for shared exposure to environmental agent(s) in determining the age and year of onset of MS in the members of each pair². The incomplete penetrance of susceptibility genes in identical twin pairs also points to the importance of environmental influences in the occurrence of disease among those with the appropriate genes.

Ebers and his colleagues express the hope that their findings "may . . . divert the search for environmental clues away from specific uncommon viruses to a broader category of events occurring early in life which are applicable to regional populations"; this comment is presumably aimed at Kurtzke and those sharing his view³ that existing epidemiological data point to a specific infectious (presumably viral) agent as a direct cause of MS. No such agent has ever been successfully linked to MS, however, either by direct isolation or by indirect (for example, serological) means, despite claims for some twenty different viruses. The epidemiological data⁴ fit equally well with a model of disease induction in genetically susceptible individuals by common viruses and mediation by a secondary process, such as autoimmunization against myelin or other brain antigens.

The situation in MS is reminiscent of that in poliomyelitis, in which there is a clear relation between the age at which viral transmission occurs and the age at which attack by the virus on the central nervous system commences. The incidence of neurological disease increases in individuals of higher socioeconomic status within a population (because this tends to be associated with viral transmission delayed to late childhood or early adolescence) and in populations living at temperate latitudes (these, in general, enjoy a better standard of living, again with later transmission of common childhood infections). A similar requirement in MS for late viral transmission has been demonstrated in a well controlled, population-based study that showed an almost tenfold increase in the risk of contracting MS as the age (up to puberty) of infection with common childhood viruses (such as measles) increased⁵.

The ability of viruses to cause autoimmunization is not in doubt. It was shown over a decade ago⁶ that measles in humans occasionally results in T-cell sensitization against myelin basic protein (MBP) and this sensitization is associated with an inflammatory demyelinating 'post-infectious' encephalomyelitis. The same relationship has been demonstrated in rats and mice infected with suitable strains of coronavirus or measles virus^{7,8}. Such

cross-immunization is usually attributed to molecular mimicry⁹. A recent report establishes that T-lymphocyte clones from MS patients specific for a single encephalitogenic MBP peptide recognize configurationally similar peptides of several common viruses equally well¹⁰. These cells are stimulated by either viral or MBP peptides to proliferate and secrete inflammatory cytokines.

The factors responsible for inducing MS, the thrust of Ebers and co-workers' contribution, must be strictly distinguished from the factors that trigger relapses (or exacerbation) of this disease once it has been induced. Here upper respiratory and gastrointestinal virus infections appear to play a key part, as first reported by Sibley *et al.*¹¹ and dramatically confirmed more recently by Panitch¹². The interval before relapse, however, is measured in days or weeks, whereas initiation of the process that leads to MS may span a period of years — Kurtzke, in his Faeroe Island studies, calculated an average interval of six years³. Also, although new T lymphocytes with pathogenic potential may be generated at each relapse, other factors may predominate, such as systemic release of inflammatory cytokines like γ -interferon, as part of the viral infection. This would result in upregulation of the MHC on vascular and glial elements in the central nervous system, which in turn would allow new lesion formation. Administration of γ -interferon in quiescent MS patients does in fact provoke symptoms of the disease, which correlate with MHC expression on circulating monocytes¹³.

Time will tell whether other (non-infectious) agents also play a role in inducing MS. The infectious/autoimmunity hypothesis has proved remarkably fruitful, but other influences, such as climate, general levels of infection and neuroendocrine processes call for deeper investigation. □

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DAEDALUS

Hard water

ENGINEERING materials must not only be strong: they must be stiff as well. Indeed, stiffness is often more important than strength. Plastics, for example, are excluded from many engineering uses not because they would break, but because they would bend.

In this connection Daedalus recalls that the stiffness of a tube can be increased by pumping a fluid through it. At a critical flow rate it even becomes infinitely stiff. Any deflection is then perfectly opposed by the internal flow. Increase the flow further, and 'negative stiffness' sets in — the pipe will react against a load. At higher flows still, oscillations occur. This is what makes a fire hose wriggle under a fast flow.

DREADCO's engineers are now exploiting these facts. Aircraft and rockets, for example, have to be very light but stiff structures; they also depend on liquid fuels pumped at high velocities. The DREADCO workers hope to achieve the first requirement with the aid of the second. Many aircraft already store their fuel in compartments in their wings, and little modification should be needed to pump it to the engines through the hollow spars of the wing structure. Indeed, this trick has already been proposed for supersonic aircraft to allow the flowing fuel to absorb frictional heat from the leading edges.

A light and flimsy aircraft or rocket, stiffened in flight by its flowing fuel, should be supremely efficient. More cunning still, its stiffness and damping could even be altered in flight by redistributing the fuel flow between different spars of the structure. The various flutters and instabilities which plague aerodynamics could be played off against each other, shifted away from dangerous resonances, or otherwise made harmless. The computing power already built into all modern aircraft and rockets could implement such strategies in real time with great aplomb.

Another application is more earthbound. Daedalus is designing a large, light building with a structural frame of plastic piping. This will be stiffened by pumping water continuously through it. Some of the water will be used by the normal domestic and heating or cooling requirements; the rest will be recirculated. The idea is to withstand earthquakes. A severe shock to the structure will stop the pumps. The building will then lose, not its strength, but its stiffness. It will absorb the battering in a flexible, rubbery manner, neither falling down nor injuring the occupants. When the danger is past, it can be pumped back to normal structural rigidity.

David Jones

The planktonic life of octopuses

SIR — The common octopuses of the world's oceans are bottom-living cephalopod molluscs. In more than half of the species known, hatchlings are planktonic¹ and swim by jet propulsion²⁻⁴. The swimming behaviour and duration of this planktonic phase before settlement have been a subject of debate. Field plankton samples very rarely contain specimens at advanced stages, and it has not been possible to determine the age of octopods using statoliths or other hard structures

undergoing appositional growth. Conversely, the only successful rearing experiment leading to settlement was made with the northwest Pacific form of *Octopus vulgaris* and indicated that the animals remain in the plankton up to the age of 33–43 days⁵.

Rearing experiments⁶ were carried out with Mediterranean *O. vulgaris* at a mean temperature of 21.2 °C to fit the inshore conditions in the northwest Mediterranean during summer, when most of the octopuses hatch⁷. At day 1 the individuals measure about 2.9 mm in total length, weigh 1.4 mg and have short arms with only three suckers each (Fig. 1). They grow exponentially, doubling their weight in roughly 8.5 days, with relative daily growth rates of 5.5–11.5%. At 60 days the settled individuals measured 18.1 mm in total length and had a mean weight of 173.2 mg (125 times their weight at hatching). The duration of the planktonic phase before settlement varied between 47 and 54 days, 10–15% of the estimated longevity of *O. vulgaris* (12–18 months)⁷. The high planktonic mortality is counterbalanced by high fecundity, which ranges from 1 to 5×10^5 eggs per female. This critical period of early life history gives this short-lived species a considerable dispersal capacity.

To quantify swimming activity, groups of individuals aged 1, 15, 30, 42 and 60 days were recorded by video, using frame-by-frame tracking at 40-ms intervals. Although the animals can turn their funnel tube in any direction for jetting, for 99% of the recorded time they jetted backwards. Relatively high swimming speeds were recorded in backwards jetting (Fig. 2). This backwards escape may allow animals in the sea to actively avoid traditional plankton nets.

It is significant that maximum jetting speed during backwards escape increased up to age 30 days, where the growth rate was highest, then declined to settlement (Fig. 2, top). This break occurred during the phase when the arms are drastically enlarged relative to the propulsive mantle, due to the highly disproportionate arm growth during the second half of planktonic life (Fig. 1). It is in this period that the animal changes shape from squid-like to the benthic octopus form. In contrast to the maximum speed plot, the relative cruising speed decreased

from 1 to 30 days of age and afterwards increased to settling, whereas cruising speed increased from hatching to settling (Fig. 2). Thus, the jet swimming of octopuses during planktonic life seems to be quite similar to the locomotion of squids, suggesting that it could enhance the dispersal capacity and colonization potential of this bottom-living species.

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Glacial cycles and orbital inclination

SIR — According to the Milankovitch theory, the 100-kyr glacial cycle is caused by changes in insolation (solar heating) brought about by variations in the eccentricity of the Earth's orbit. There are serious difficulties with this theory: the insolation variations appear to be too small to drive the cycles, and a strong 400-kyr modulation predicted by the theory is not present. Moreover, the amplitude of the glacial cycle has been large at times (400 years ago and today) when the eccentricity modulation has been near zero; this conflict is also called the 'Stage-11 problem'¹. In addition, improved measurements have uncovered an apparent causality problem: the sudden terminations of the glacial cycles appear to precede the increases in insolation^{2,3}, although this interpretation has been disputed⁴. We suggest that a radical solution is necessary to solve these problems, and we propose that the 100-kyr glacial cycle is caused, not by eccentricity, but by a previously ignored parameter: the orbital inclination, i , the tilt of the Earth's orbital plane.

Ancient climate is recorded in sediment through change in the oxygen isotope ratio, $\delta^{18}\text{O}$, which is believed to

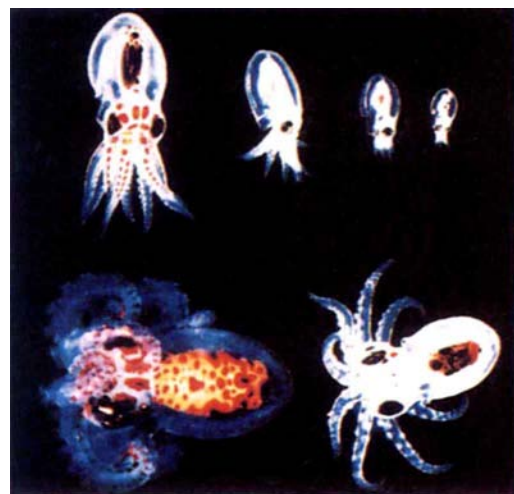


FIG. 1 Reared individuals of *O. vulgaris* at ages 1, 20, 30, 42, 50 and 60 days (upper right to lower left). The size expressed in dorsal mantle length (ML, distance from the mantle end to the midpoint between eyes) is 2.0, 3.0, 4.3, 5.9, 6.6 and 8.5 mm, respectively. Individuals were photographed under anaesthesia, at the same scale.

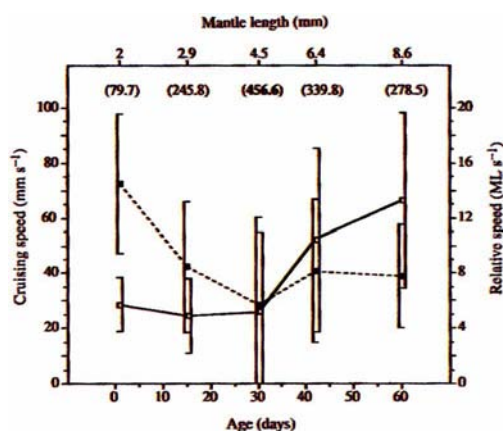
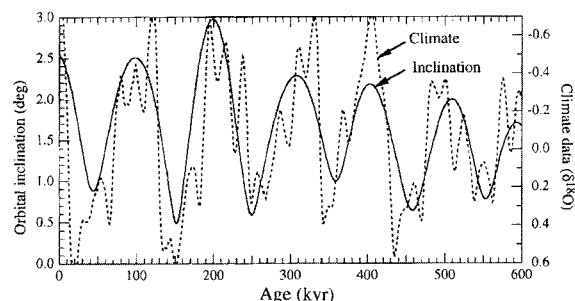


FIG. 2 Cruising speed during swimming (solid line) and relative swimming speed (dashed line) are plotted against age and dorsal mantle length for *O. vulgaris* individuals aged 1, 15, 30, 42 and 60 days, giving mean and standard deviation. Values in parentheses in the top part are maximum swimming speeds (in mm s^{-1}). Data were collected from digitized video records of groups of five individuals, filmed in still water in a circular tank 250 mm in diameter, with a water level of 30 mm. Settled individuals aged 60 days swim only when gently disturbed (crawling speed on the ground $\sim 12 \text{ mm s}^{-1}$).

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Comparison of orbital inclination (solid line, lagged by 33 kyr) and $\delta^{18}\text{O}$ climate data (dotted line) from SPECMAP⁵.

reflect the percentage of the Earth's water frozen in ice. The figure shows $\delta^{18}\text{O}$ (dotted line) for the past 600,000 years from the SPECMAP compilation of data from five sea-floor sediment cores⁵. The figure also shows the orbital inclination (solid line), calculated by direct integration of planetary perturbations⁶, transformed to the invariable plane (the plane of symmetry of the Solar System), and shifted to give the best least-squares fit to the $\delta^{18}\text{O}$ data. (Only three parameters were adjusted, one for the delay and two for the overall scale.) For the best fit, i preceded $\delta^{18}\text{O}$ by 33 ± 3 kyr; as this is positive, there is no causality problem. Similarly, the presence of a strong variation in i near 400 kyr solves the Stage-11 problem.

The existence of the 100-kyr cycle of orbital inclination does not seem to have been noticed previously by climatologists or astronomers. It may have been missed for two reasons. Ever since Milankovitch, the implicit assumption has been that

insolation is the driving force for climate cycles, and insolation is not directly affected by orbital inclination. Second, the 100-kyr cycle is not evident when i is calculated in the usual reference frame based on the present orbit of the Earth. Only when transformed to the invariable plane (or a plane near it) does the 100-kyr cycle unmix from the obscuring effect of a strong 70-kyr orbital precession cycle. We note that a 70-kyr cycle has been reported in $\delta^{18}\text{O}$ data from other sedimentary samples⁷, and we suggest that this cycle may be related to orbital precession.

The only mechanism we have found that could link orbital inclination to climate is extraterrestrial accretion of meteoroids or dust. Such material can be detected in ice and sedimentary rock by analysis of iridium; Walter Alvarez has pointed out that extraterrestrial dust cycles could be detected using ^3He . If this mechanism is correct, a 100-kyr cycle should be seen in ice and sediment records of extraterrestrial accretion.

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Uncertainty in ancient phylogenies

SIR — Use of phylogenetic methods to estimate ancestral phenotypes is becoming widespread in evolutionary biology¹. For example, Jermann *et al.*² estimated and then synthesized ribonucleases of early artiodactyl ancestors, beautifully demonstrating the power of these methods for elucidating molecular evolution. Essential to and frequently missing from such studies is a measure of the statistical uncertainty of estimated ancestral states needed to gauge their reliability. I have used a maximum-likelihood (ML) method to estimate the amino acid at position 38 of artiodactyl ribonuclease, the residue found² to be most crucial to enzyme catalytic activity³.

The method applies the Markov model of trait evolution³, assuming that the rates of change between states are constant through time and over all branches of the phylogenetic tree. A given trait may have

two states, i and j . The transition rate from state i to state j over an infinitesimally short time period is q_{ij} . This parameter and its converse, q_{ji} , can be estimated directly from data on modern species and their phylogenetic relationships. The ML estimates for the rates³ are obtained by maximizing:

$$L(q_{ij}, q_{ji}) = \sum_{i=1}^m \sum_{j=1}^m \sum_{n=1}^n (P[S_1, S_2, \dots, S_m, X_1, X_2, \dots, X_n])$$

The term in parentheses on the right is the probability of arriving at the given trait values S of the m modern species when trait values at the n interior nodes (ancestors) are $X_1, X_2, \dots, X_n$ (ref. 3). This equation can also be used to compute likelihoods of alternative states for any single ancestor as the portion of the sum contributed by each state at the given node. The state having highest likelihood is the ML ancestor state, conditional on the estimated values of q_{ij} and q_{ji} . The ratio of the two likelihoods measures the level of support for the ML estimate⁴. The procedure is similar for traits having three or more states³. The method evaluates

each ancestor in turn, and while doing so assumes that possible states at remaining nodes have equal prior probability.

To simplify the analysis, I modelled only two states at position 38 of artiodactyl ribonuclease, Gly and Asp (denoted G and D in Fig. 1 of ref. 2), and I deleted the two species (nilgai and impala) having rarer amino-acid residues (Asn and Ser). This simplification does not affect ML ancestral states because the rates of transition between Gly or Asp and the rarer residues were estimated to be small, such that the likelihood of the rarer states in the ancestors was also small. It also does not significantly alter conclusions concerning the levels of support for Gly and Asp in the ancestors.

Computations were carried out using DISCRETE (ref. 3) and a reduced program developed independently. Maximum likelihood yielded $q_{\text{Gly, Asp}} = 0.0164$ and $q_{\text{Asp, Gly}} = 0.0102$. Thus, the half-life of Gly (the time interval over which a lineage in state Gly has a 50% chance of changing to state Asp) was 42 Myr, and the half-life of Asp was 68 Myr. Total tree length was 450 Myr (ref. 2); change at residue 38 is therefore not expected to be rare on this tree. These estimates are derived solely from the data on position 38 in artiodactyls rather than from data on all sites or for proteins in general⁵. This avoids the unnecessary assumption that transition rates and states at position 38 of artiodactyl ribonuclease are typical of sites on this or other proteins.

Asparagine was estimated to be the most likely state in all ancestral artiodactyls with one exception (the immediate ancestor to the two camel molecules, which was estimated to be Gly). This contrasts with the results from parsimony² which estimated that Gly was the residue at position 38 of the three most ancient species. More significantly, the uncertainty of the ML estimates for these three ancient nodes was high; likelihood ratios for all three were less than 1.4. By comparison, support limits for a ML estimate (analogous to 95% confidence intervals) generally encompass all values whose $\ln(\text{likelihoods})$ are within 2 units of the maximum⁴, corresponding to a likelihood ratio of $e^2 = 7.4$. Similarly, a likelihood ratio of 6.82 (corresponding to a $\chi^2_1 = 2 \ln(6.82) = 3.841$) is required to reject a statistical null hypothesis⁶. Consequently, both states for early artiodactyls, Gly and Asp, are highly compatible with the data from contemporary species. In particular, a transition from Gly to Asp between the ancestors g and h is not supported by the likelihood analysis.

Variations on the above method are possible that differ in the degree to which likelihoods for a given state are conditional on the values of additional parameters in the likelihood model. For example, the states of all ancestors may be estimated

¹ J. Felsenstein (University of Washington) has independently developed a similar method for estimating ancestral nucleotide sequences at interior nodes of a ML phylogeny and Z. Yang (Pennsylvania State University) has recently developed an alternative bayesian approach.

simultaneously by choosing the single combination of ancestral states making up the largest portion of the sum, *L*. Support for the ML estimate at a given node is then evaluated by comparing its likelihood with that of the alternative state at the same node computed using the most likely arrangement of states at remaining nodes. The results were similar: Asp was the most likely state in early artiodactyls; likelihood ratios for the three ancients **h**, **i** and **j** were <2.6. I also tried another procedure in which the transition rates $q_{Gly,Asp}$ and $q_{Asp,Gly}$ were no longer fixed, but could vary depending on the state of the ancestor of interest. The residue at a given node was set to Gly, and the ML estimates for transition rates were then recomputed to best accommodate that ancestral value before obtaining the corresponding likelihood. These steps were repeated with the same ancestor set to Asp. Asparagine was again the most likely state in all three oldest ancestors, but the likelihoods were less than 1.4 times better than those for Gly.

These results used data from the artiodactyls² alone, whereas the parsimony reconstruction included information from older branches⁷. For example, whales are the sister taxon to the artiodactyls^{7,8} and the one species surveyed has Gly at position 38 (ref. 7). Adding this species did not change the likelihood results for the earliest artiodactyls, presumably because a single branch ~55 Myr provides little information. Asparagine was again the most likely state in the three early artiodactyls and support remained low (<1.7). The results were also little changed when horse and rodents were added as lower branches. Addition of these taxa confirm that rates of change between Asp and Gly are relatively frequent: the horse has Gly whereas rodents include some species with Gly and others with Asp⁷ (the casiragua with Glu was deleted from this analysis). Uncertainty of states in early artiodactyls is thus little diminished by the addition of these other taxa. In another analysis, pancreatic ribonuclease alone was used, the protein sequence from bovine seminal plasma being deleted. ML estimates and level of support for remaining ancestors **i** and **j** were little affected.

The above calculations assume that the

phylogenetic tree of relationships among artiodactyls⁷ is correct, including branch lengths, but the phylogenetic tree is itself merely an estimate. It should be possible to incorporate likelihoods of alternative trees into the calculation of ancestral states. Such a procedure might produce ML estimates different from those presented above, but it is unlikely that levels of support will be much improved. Uncertainties over ancient residues at position 38 of ribonuclease are high in large part because of a relatively high rate of transition between alternative states coupled with long spans of time. These are undoubtedly features of the correct phylogenetic tree, since variation at position 38 is seen at all levels of evolutionary relationships among taxa⁷.

Perhaps the greatest weakness of the above method is the assumption that rates of evolution are constant throughout the tree. In truth, these may differ from lineage to lineage and through time. Its advantage is that methods can be devised to test the assumption of constant rates, and also to fit more complex models in which rates are allowed to vary, although many attempts to do this here did not reduce the uncertainty of ancestor estimates.

These results show that estimates of ancient ribonuclease sequences are highly uncertain, at least at the most critical position 38. More generally, they show that ancient sequences can be estimated in a probabilistic framework, and that uncertainty of estimates can be quantified. Such information will be valuable when designing studies to reconstruct ancient molecules or other characteristics of early ancestors.

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BENNER *ET AL.* REPLY — We agree that maximum likelihood (ML) methods are valuable for reconstructing ancient forms of life. We do not agree, however, that such methods are “frequently missing from such studies”. In 1992, the Computational Biochemistry Research Group at the ETH in Zürich produced a tool (DARWIN) that makes automatic ML reconstructions⁹. DARWIN yields prob-

abilistic ancestral sequences where each residue is represented as a vector of unit length in 20 dimensions¹⁰. The components of the vector in each dimension reflect the probability that each of the 20 natural amino acids was present at this position in the ancestor. We have used DARWIN to reconstruct some 50,000 amino acids encoded by the proto-genome¹¹ (the most recent common ancestor of archaeobacteria, eubacteria and eukaryotes¹²), and to propose a model for the metabolism of this ancestor¹³.

Nor do we agree that ML “estimates of ancient ribonuclease sequences are highly uncertain.” In the ancestral ribonuclease near the divergence of the brain, seminal and pancreatic ribonucleases (corresponding in the fossil record approximately to the origin of ruminant digestion), DARWIN reconstructs a Gly at position 38 with 99.5% certainty if all available ribonuclease sequences are considered⁷. Over the entire ancestral sequence, DARWIN assigns 118 of the 124 residues with >95% probability. Only one residue (at position 102) is assigned with a probability below 50% (ref. 2).

Why are Schluter's conclusions different from those of DARWIN? It is difficult to say from the information available. Differences in reconstructions are most often traced to different connectivities in the underlying evolutionary tree, which need not be clearly defined. DARWIN allows maximum likelihood factors to influence the positions and lengths of branches of that tree. The ML analysis discussed by Schluter is only concerned with the sequence variation, and assumed a tree generated by parsimony methods. We are not sure of the implications of such a hybrid approach. All we can say is that DARWIN, using a ML approach, consistently yields an ancestral reconstruction at position 38 not remarkably different from those yielded by consistent application of parsimony, even though the preferred connectivity of the evolutionary trees differs. If one moves away from the point of transition (Gly 38→Asp 38), DARWIN places a Gly at position 38 in more ancient sequences with only low statistical uncertainty and Asp in more recent sequences, again with low uncertainty. If the remaining uncertainty is unacceptable, other ancient sequences could be prepared and studied (as in ref. 2), or additional data collected to define the tree more precisely.

The most important point to be made by recent work in palaeomolecular bio-

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

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chemistry, however, is that molecular reconstructions should be the beginning of an experimental research programme and not the final goal of an evolutionary analysis. When reconstructed ancient biomolecules are made and studied in the laboratory, they can provide information about ancestral environments¹⁴⁻¹⁶, help correlate *in vitro* biochemical behaviour with *in vivo* physiological function², and uncover mechanisms by which organisms generate new biomolecular function.

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A large French Cretaceous bird

SIR — We report the discovery of a fragment of the synsacrum of a large bird in the continental Upper Cretaceous of south-eastern France. It is the first bird to be reported from the Upper Cretaceous of southern France. Although no precise identification is possible, it was apparently a bird with an advanced synsacrum, possibly as large as a modern cassowary or ostrich. This discovery suggests that some of the abundant Late Cretaceous fossil eggs from southern France usually assigned to dinosaurs may in fact be bird eggs.

The 65-mm-long synsacrum fragment was discovered by two of us (P.M. and A.M.S.) in a Late Cretaceous (probably

Early Maastrichtian¹) vertebrate locality in the Fox-Amphoux basin (Var department, Provence). Its fairly well preserved ventral part allows a relatively accurate placement by comparison with synsacra of modern birds. It is broadest anteriorly, with three laterally and dorsally directed transverse processes, which originally contacted the ilium, on the right side (only two are preserved on the left side). In this region, the dorsal part has a system of complicated, poorly preserved, bony laminae which correspond to the modified neuropophyses occupying the roof-shaped space formed by the dorsally fused ilia in modern birds. More posteriorly, the ventral part of the specimen becomes narrower, forming a parallel-sided rod with a shallow median furrow. Dorsally, this part shows the poorly preserved bases of vertically directed processes. Comparison with modern birds shows that this fragment corresponds to the posterior synsacral lumbar vertebrae and anterior sacral vertebrae².

The attribution to a bird is based on the complete fusion of the vertebral elements, which is exactly comparable to the condition in modern birds. In theropod dinosaurs with avian characters, such as ornithomimosaurs, oviraptorosaurs, troodontids and dromaeosaurids, vertebral fusion in the sacrum is never so pronounced and the limits between the individual vertebrae are still visible. Many primitive Cretaceous birds³ also display incomplete fusion of the synsacral elements. Although neither a definitive identification nor an attribution to one of the main groups of Cretaceous birds recently recognized (enantiornithines, ornithurines and "transitional shorebirds")⁴ is possible, our specimen apparently indicates a form with an advanced

synsacrum resembling that of modern birds. It is the first bird skeletal element to be described from the Upper Cretaceous of France and one of a very few birds known from the European Upper Cretaceous. Bones from the Maastrichtian of Transylvania once referred to birds are now considered as those of small theropods. Bird fragments from the Chalk of Scandinavia do not include sacral elements.

Although it is difficult to estimate the total length of the synsacrum, because the proportions of this element vary quite widely among birds, the great breadth (maximum breadth, as preserved, 40 mm) and robustness of the specimen are remarkable. The sacrum of the Fox-Amphoux bird was more robust than that of *Hesperornis regalis* (hitherto the largest known Cretaceous bird, with a total length of 1.80 m), and differently built. Comparisons with recent birds suggest that the synsacrum from Fox-Amphoux was in the size range of the cassowary or even the ostrich (although there are no special morphological resemblances with living ratites). The specimen is also somewhat reminiscent of the giant Eocene flightless bird *Diatryma*. Although the proportions of the complete skeleton cannot be reconstructed, there is no doubt that it was indeed large, especially by Cretaceous standards. Whether it was a flying or flightless form cannot be determined.

The discovery of a large bird in the Upper Cretaceous of Provence has implications for the interpretation of the abundant fossil eggs from the Upper Cretaceous of southern France, which have usually been attributed to dinosaurs⁵. No embryo or neonatal remains have yet been found in association with these eggs, and the occurrence of a large bird in the Upper Cretaceous of Provence suggests that some of them may in fact be bird eggs. This in turn implies that stratigraphical or palaeobiological speculations based on the assumption that all those eggs are dinosaur eggs should be treated with caution.

Eric Buffetaut

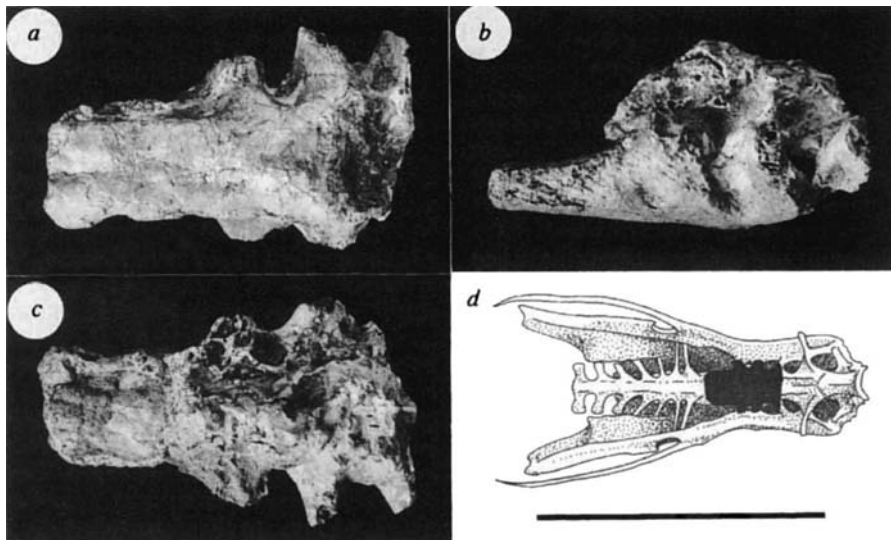
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Synsacrum fragment of a bird from the Upper Cretaceous of Fox-Amphoux (Var, France; deposited at the Musée des Dinosaures, Espéraza) in ventral (a), dorsal (b) and right lateral (c) views. Scale bar, 5 cm. Photos by C. Abrial. The sketch (d) shows the approximate position of the fragment in a bird pelvis, seen in ventral view (based on a flamingo pelvis, although no special relationships with flamingoes are implied). Drawing by G. Le Roux.

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Family fortunes

Leslie C. Aiello

Ancestral Passions: The Leakey Family and the Quest for Humankind's Beginnings. By Virginia Morell. Simon and Schuster: 1995. Pp. 638. \$30, £20. To be published in the United Kingdom in January.

THE past 12 months has seen the announcement of two new species of early hominid, *Ardipithecus ramidus* from Aramis in Ethiopia (see *Nature* 371, 306; 1994 and 375, 88; 1995) and *Australopithecus anamensis* from the Lake Turkana region of Kenya (*Nature* 376, 565; 1995). Both these new species are more than 4 million years old and push the fossil evidence for the origins of the human line closer to the biochemically inferred date of 5 to 6 million years ago. They also suggest that the idea of one evolving line of hominids during that time interval may be too simplistic for the evidence. New foot fossils from the South African australopithecine site of Sterkfontein (*Science* 269, 521; 1995), dated to more than 3 million years ago, also support the idea of evolutionary experimentation during the first few million years of human prehistory. The Sterkfontein foot indicates that there may have been other types of two-footed walking characteristic of these early hominids and that these most probably did not occur in a single linear sequence.

These ideas are causing considerable excitement among palaeo-anthropologists. But when we step back from the frenzy of discovery, we see that the new fossils and the emerging controversy over their interpretation are merely the most recent in a long trail of discovery and heated controversy that spans the past 70 or so years of fossil hunting in Africa. We also realize that much of what is happening in the field of human evolution today can be fully understood only in the context of the history of the field, the fortuitous nature of discovery of hominid fossils and the few strong personalities dominating their discovery and interpretation.

There is no shortage of biographies or popular accounts of discovery that cover the history of human palaeontology in East Africa, but these tend to be personal accounts of individual involvement in the field. *Ancestral Passions*, the newest contribution to the literature on the history of human evolution in East Africa, has a different purpose. By chronicling the history of the Leakey family's involvement in East African human palaeontology, it provides a much broader scope than much of the earlier literature. It shows us how the lives

and careers of some of the main players involved with the latest new discoveries and controversies (Meave Leakey, Don Johanson, Tim White, Alan Walker and so on) have been intertwined with the Leakey family. It allows us to appreciate how much of our current knowledge about human

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REASONS

Leader of the hominid gang: Louis Leakey (1903–1972) and the type specimen *Homo habilis*, or 'handy man', discovered at Olduvai Gorge, Tanzania, in 1960.

origins in East Africa is dependent on the influence of this single family and on the passions that have driven the Leakey's over the years. *Ancestral Passions* also differs in another way from earlier biographical works. Because the author is a journalist rather than a researcher in the field of human evolution in East Africa, she has no personal story to tell or cause to fight.

Ancestral Passions lacks the sensationalism and scandal that has propelled some books on human origins into the public spotlight. Instead it adopts a balanced and matter-of-fact tone that puts the reader in the role of a fair witness to the unfolding events. The 554 pages of text are carefully researched, drawing on previously published sources, extensive archival material and personal interviews with both key and more peripheral people. Points are expanded in welcome footnotes, and sources are meticulously documented in 50 pages of endnotes, themselves a valuable

resource for students of the field.

The book contains few big surprises for specialists already familiar with the Leakeys and the outline of the Leakey story — Louis Leakey's entrepreneurial show-business manner, Mary Leakey's meticulous archaeology and often tetchy nature and Richard Leakey's rivalry first with his father and then with Don Johanson. It does, however, provide a valuable introduction to the earlier periods of human evolution in East Africa and contains enough gems of information to keep the specialist awake and interested. One of my favourites is Louis Leakey's original name for Olduvai Hominid 5, the famous robust australopithecine fossil from Olduvai Gorge that he finally published as *Zinjanthropus boisei*. The original name was *Titanohomo mirabilis* ('wonderful titan-like man'). Another is the explanation of why Charles Boise, in whose honour *Zinjanthropus boisei* was ultimately named (and who established the Boise Foundation, which continues today to fund research into human origins), became a benefactor of the Leakeys. It was all down to national pride. The original donations in 1948 were designed to keep the Miocene research in East Africa "under British auspices" by providing the Leakeys with the means to compete successfully in the field with a well-equipped US expedition headed by the entrepreneur Wendall Phillips. The prize at that point was to obtain a skull or skulls of a fossil ape from rich deposits in western Kenya. The Leakeys won with the discovery on Rusinga Island of the skull of *Proconsul africanus* (now *P. heseloni*).

The main impression one gets from reading *Ancestral Passions* is a strong sense of continuity leading from one East African fossil discovery to another, discoveries that each, in their time, radically changed our ideas of human evolution. Through all this is the pervasive presence of the Leakey family, whose interest determination and drive were responsible for, or had in one way or another a large influence on, the course of events. The excitement of discovery and the controversy over interpretation have not changed over the years. If anything has changed, it is only that current interpretations are less impulsive and more scientifically based than those that in particular characterized Louis Leakey's early career. There is no doubt that human evolution is a dynamic field and that in the future new discoveries will cause controversies and upheavals in interpretation to rival those of today and those of the past. One sure thing, though, is that our current understanding of early human evolution in East

Africa would undoubtedly be much different if Louis Leakey had failed to survive his difficult and premature birth 92 years ago and the Leakey family had never existed. Morell should be congratulated for bringing this all so clearly and competently to our attention. □

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How we got here

Chris Stringer

The Fossil Trail: How We Know What We Think We Know about Human Evolution. By Ian Tattersall. Oxford University Press: 1995. Pp. 276. £17.99, \$25.

WRITING popular books on human evolution is a difficult business these days. For one thing, there is a lot of competition in the market that is regularly replenished. For another, events can move fast (see previous page). But the major stumbling-block is the problem of finding a new approach or, at least, a new angle to the story. A common approach is the historical one, showing how the subject has gradually developed through ideas and discoveries. Another is to concentrate on present debates at the expense of historical development. Ian Tattersall has tried to combine both approaches to show how received wisdom can shape interpretation and how what we expect to find influences our understanding of what we do find in the fossil record.

Tattersall has to go over some familiar ground with early influences such as Boucher de Perthes, Lamarck, Cuvier and Darwin, but he manages to find some fresh gloss to apply to his accounts of the Neanderthal, '*Pithecanthropus*' and *Australopithecus* discoveries. Only occasionally does he fail to update some of the data he introduces here — for example, the Aurignacian is now dated well beyond 32,000 years at several European sites, and the Alpine glacial-interglacial stage names have generally been abandoned away from their type areas.

Tattersall's views come through strongly, and can be nicely encapsulated in this extract from his discussion of multi-regional evolution:

This viewpoint does seem to me to illustrate, better than any other current example, the extreme parochiality with which paleo-anthropology is cursed. Up to this point, it appears, we paleoanthropologists have proven unable as a group to shed our major historical burden: the birth of our science out of the study of human anatomy, rather than out of the comparative anatomy and geology from which other areas of vertebrate paleontology emerged. Under the

dead hand of this heritage, which places our species at the center of the academic universe, we seem incapable of seeing *Homo sapiens* as simply one more species among many. We constantly seek special explanations for ourselves. And, perhaps worst of all, we are afflicted with a tradition of looking for variability in the collections of fossils, rather than for diversity.

Couple this with an historical bias towards gradual evolutionary change, and it quickly becomes clear why Tattersall is so disenchanted with many aspects of mainstream palaeoanthropological thinking.

The debate on species recognition in fossil hominids is one in which Tattersall is actively engaged, and one of his consistent themes in the book is how, ever since the naming of *Homo neanderthalensis* by William King in 1864, hominid species have been named and then lost at a fairly steady rate, thus keeping the number of recognized species at a low level. His feeling, which I share, is that the three generally accepted species of the genus *Homo* — *habilis*, *erectus* and *sapiens* — are certainly an underestimate of the number that really existed. Each of these species can probably be split into two at least, and the conventional early hominid story is probably also an oversimplified one in terms of recognized species, if not of genera.

But Tattersall also points out, quite appropriately, that closely related species may differ little in terms of bony or dental morphology (the only aspects likely to have fossilized) and argues that we should concentrate on the variation *between* modern species as a yardstick for fossils rather than the variation *within* them. His argument is that if distinct 'morphs' can be recognized in fossil remains, then different species are indeed present. In principle this sounds fine, but the problem is that, for example, the crania of North American Inuit can be fairly consistently distinguished from those of Native Australians and yet these are both indubitably members of one species, *Homo sapiens*. In practical terms, I find it difficult to apply the morph rule except in the most extreme fossil cases, such as Neanderthals and Asian *Homo erectus*, where really distinct and surely specific patterns do exist. In the late Middle Pleistocene of Africa and Asia, for example, the morphs seem much fuzzier.

Nevertheless, Tattersall's approach is surely more realistic than the extreme lumping tendencies that have predominated over the past hundred years, and it would be nice to think that this "refreshingly opinionated" book (to quote the cover) will have a lasting influence on the next generation of palaeoanthropologists. □

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Children's Books

Nature plans to publish on 16 November a supplement in which children's books and software and software will be reviewed. The supplement will cover science books and software for children of all age groups.

Publishers are invited to send suitable material for consideration, taking note of the following criteria:

- Only books and software issued in 1995 will be considered;
 - Books and software dealing with any aspect of science, technology, medicine, natural history or the environment are eligible (including encyclopaedias, dictionaries and games), although school curricula texts are excluded;
 - The main language used must be English;
 - If possible, cross-platform software (both Macintosh and PC) should be provided.
- Deadline for submission is 22 September. Publications for review should be sent, together with details of price and availability, to Peter Tallack, Book Review Editor, *Nature*, 4 Crinan Street, London N1 9XW, UK (tel: +44 (0)171 843 4567; fax: +44 (0)171 843 4596/7; e-mail: p.tallack@nature.com).

New in paperback

Newton's Clock: Chaos in the Solar System by Ivars Peterson. W. H. Freeman, £11.95. A readable popular history of the relationship between mathematics, astronomy and humankind's desire to understand the Solar System.

Control of Crop Diseases by W. R. Carlile (2nd edn). Cambridge University Press, £12.95. This brief introduction to modern disease control in commercial crops has been updated to include changes in practices and legislation that have come into effect since the first edition was published in 1988. Also includes a new chapter on the detection and identification of disease, with emphasis on molecular diagnostic techniques.

Gene Cloning: An Introduction by T. A. Brown (3rd edn). Chapman and Hall, £17.99. This well established introductory text, which is aimed at undergraduates and those who have little or no previous experience of cloning techniques, now contains new chapters on the polymerase chain reaction and plant molecular biology.

Enrico Fermi, Physicist by Emilio Segre. University of Chicago Press, \$13.95, £11.25. "Authoritative, revealing and inspiring," said *Nature's* reviewer of the book when it first appeared in 1970. "It covers in a masterly fashion the most exciting thirty years of modern physics and the character and activities of one of its greatest contributors".

How to Write and Publish a Scientific Paper by Robert A. Day (4th edn). Cambridge University Press, £12.95. Gives sensible advice on all manner of subjects, including how to write book reviews.

The populist gene

John Durant

The DNA Mystique: The Gene as a Cultural Icon. By Dorothy Nelkin and M. Susan Lindee. W. H. Freeman: 1995. Pp. 276. \$22.95, £15.95.

IN his book *The Extended Phenotype*, Richard Dawkins tells the story of a lecture during which a member of the audience became tearful at the prospect that some behavioural differences between men and women might be "genetically determined", and thus (she supposed) inevitable. The point of the story is that genetic influences on behaviour are in principle no more or less likely to be alterable than other (for example, environmental) influences; but somehow many people have come to believe that genes exert a uniquely powerful influence on our lives.

In *The DNA Mystique*, Dorothy Nelkin and Susan Lindee recount a 1993 episode of the American television programme "Law and Order", in which a young man was tried for murder. His defence attorney invoked in mitigation the fact that his client possessed the chromosomal aberration *YYY*; but in the event the accused man himself requested that he be given the maximum sentence: his situation, he said, was hopeless, because he was "genetically predisposed to kill".

The DNA Mystique is a full-length sociological study of the phenomenon that so intrigued Dawkins. Nelkin and Lindee are interested in the powers that are ascribed to the gene in American culture. For them, the gene is not only a biological entity but also "a cultural icon, a symbol, almost a magical force". Insisting that DNA is a molecule with social as well as biological properties, they set out to chart its presence in popular culture through the analysis of what they term "a vast, quixotic data-base" embracing not just magazines and television programmes but also cartoons, jokes, musical lyrics, radio talk shows and much more.

In essence, the story that Nelkin and Lindee tell is rather simple. For 25–30 years after the Second World War, a form of liberal environmentalism was the norm in American academic and popular culture; but over the past two decades a new form of hereditarianism has emerged in both arenas. Gaining credibility from the extraordinary advances in the field of human molecular genetics, a doctrine that Nelkin and Lindee dub "genetic essentialism" has

come to exercise increasing influence in American public life.

Genetic essentialism, we learn, "reduces the self to a molecular entity, equating human beings, in all their social, historical, and moral complexity, with their genes". Nelkin and Lindee trace the influence of this doctrine in the advertising industry, movie-making and American soap operas, as well as in apparently more serious public debates about everything from the nature of homosexuality and the alleged collapse of American family life to racial and gender differ-

become so much more than a mere macromolecule.

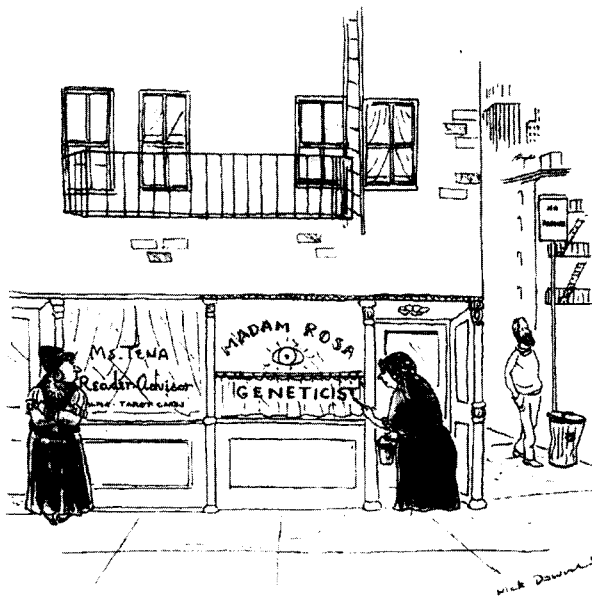
This having been said, I have two major reservations about *The DNA Mystique*. The first has to do with the nature of the evidence on which the analysis rests. Nelkin and Lindee are refreshingly frank about the eclectic nature of their "vast, quixotic data-base", as well as about the fact that theirs is "not... a statistical study but an analysis of folklore". But to describe everything reported in this book as folklore is, I think, stretching things a little. At one extreme,

there are serious academic articles aimed at establishing scientific claims; and at the other there are cartoons (such as the memorable "Madame Rosa, geneticist") many of which appear to be ironic or even satirical in intent. It is not always clear what all these extremely heterogeneous cultural artefacts have to tell us about the status or the significance of DNA in popular culture.

My second, related worry has to do with the explanatory role of the concept of 'genetic essentialism'. This rather abstract concept is sufficiently general that it can be laid over an enormous range of cultural products; but once again, I doubt that everything cited or quoted here is really working to the same intellectual or ideological end. In many ways,

The DNA Mystique hovers uneasily between a liberal-environmentalist critique of genetic essentialism (of the sort that the United States has produced in considerable numbers over the past couple of decades) and a sociological analysis of genetics in popular culture. It is the latter, in my view, that is by far the more interesting and important project; and although they have clearly not said the last word, Nelkin and Lindee are to be warmly congratulated for opening up this intriguing field to further study. □

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From *The DNA Mystique*

ences and the nature of criminality.

Nelkin and Lindee acknowledge that genetic essentialism can serve many different social agendas; but they seem to find it being used almost everywhere to buttress the ideology of individualism. However, where once individualism was an energizing doctrine of personal hope, today it is often an enervating form of genetic fatalism.

As the authors put it: "In the 1990s, the popular rhetoric of DNA is as much about loss of control and acceptance of biological fate as it is about cure and the control of our future". Hence, presumably, the extraordinary new kind of practical aid — the 'don't help yourself' book, devoted to helping people to accept their natural limitations. Nelkin and Lindee are right to insist that the multiple cultural uses of genes and genetics are worthy of study in their own right. At a time when the term DNA is used not only in biology but also in the cosmetics industry (as the name of a perfume), in children's comic writing (*The DNA Agents*) and in a proposal for a new line of stationery (DNA cards, containing a piece of one's favourite star's DNA, courtesy of the polymerase chain reaction), it is clearly important to understand the many ways in which the double helix has now

New Journals

Next week's issue contains *Nature's* New Journals review supplement which aims to provide readers and librarians with guidance and comment on a range of new publications launched over the past 18 months or so. Journals under consideration this year include *Journal of Consciousness Studies*, *Artificial Life*, *Toxicology and Ecotoxicology*, *Nature Structural Biology*, *Immunity*, *Journal of Computational Neuroscience*, *Journal of Laser Applications and Meteorological Applications*.

Ancient light

Owen Gingerich

Cosmology in Antiquity. By M. R. Wright. Routledge: 1995. Pp. 201. £40, \$59.95 ((hbk); £12, \$17.95 (pbk).

DOES time exist in the absence of matter? Can the great variety of the world around us be explained in terms of a few elemental entities? Are these ultimate entities expressible in mathematical terms? Was the Universe created by a supranatural agent, or can it be understood in a self-contained mechanistic fashion?

These questions, which continue to exercise cosmologists today, were first asked by ancient Greek thinkers. As Wright emphasizes in this succinct and nicely organized account, the Greek philosophers were struggling to construct a new vocabulary that could come to terms with such abstract queries. It is fascinating to see Lucretius coping with the question of whether the Universe can have an edge, or Parmenides inquiring about whether it could have a beginning: "I will not allow you to say or think that it comes from what is not, for 'is not' is unsayable and unthinkable. And what necessity would compel it to emerge from a starting-point of nothing at a later rather than an earlier time?"

Plato argues brilliantly that there can be no time outside the cosmos: "Time is the number of motion — but there is no motion without physical body.... Therefore it is clear that no place, void, or time is outside [the cosmos]; and that is why the things there are not such as to be in a place, nor does time make them age... but, free from alteration and suffering, in possessing the best and most independent life they continue the whole of eternity."

In presenting these ideas, Wright first systematically reviews the often sparse and fragmentary sources, and then in subsequent chapters approaches the material topically, with chapters ranging from "Models, Myth and Metaphors" to "The Cosmos and God". This creates a modest amount of repetition, but since

for many readers the material will be unfamiliar, the duplication will be an advantage.

By entitling her book *Cosmology in Antiquity* rather than *Cosmology in Greek Antiquity*, Wright implies that there is no cosmology worth talking about in the other ancient cultures. This may well be true, but the Greek achievement would have been set off in richer dramatic relief if she had more specifically contrasted the Aegean origins of modern science with the surrounding matrix of ancient Egypt and Mesopotamia, as done, for example, in the classic *Before Philosophy* by Henri Frankfort *et al.*. Certainly the invention of mathematical science was already taking place in ancient Babylon, even if evidence for cosmological thought is lacking. This difference, then, is worth a sharper delineation than Wright gives.

It is clear that Wright's strength lies in her linguistic subtlety and her command of the changing vocabulary as the Greek thinkers come to terms with such notions as origin, eternity and soul. Her footing is less sure on the technical scientific issues. An example is the caption to Figure 9: "The outer sphere containing the fixed stars revolves with the axis of its poles at an angle to that of the earth's diameter." (The figure itself compounds the confusion.) When she mentions that Plato

believed that the basic elements could be constructed from scalene and isosceles triangles, we could wish for more explanation. Similarly, Plato's ordering of the planets from the numbers 2, 3, 4, 8, 9 and 27 passes without notice. And in her urge to bring modern relevance to these ancient questions, I think she rather stretches our patience in the comparison of Empedocles's elements of earth, air, fire and water with the four nucleic-acid bases of DNA.

Nevertheless, within the intentions of the "Sciences of Antiquity" series to which this brief book belongs, it is a successful and useful compendium, a handy source on ancient Greek cosmology and an excellent supplementary textbook for courses dealing with the origins and development of modern science.

The book's designer deserves commendation for placing the many inset quotations in the same legible size as the body text, particularly because they are such an essential part of the presentation. Meanwhile, some hapless editor rates a zero for using a word processor to change Ptolemy's *Almagest* to *Amalgest* with global consistency. □

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This slippery character appears in *The Dangerous Snakes of Africa* by Stephen Spawls and Bill Branch (Blandford, £20). As well as providing a well illustrated species-by-species guide, the book contains vital information on the effects, diagnosis and treatment of snakebite. For more general readers, Blandford has also just published *The Encyclopedia of Snakes* by Chris Mattison, a comprehensive and colourful reference book covering all aspects of snake biology. Price is £25.



Contrasting properties of two forms of long-term potentiation in the hippocampus

Roger A. Nicoll & Robert C. Malenka

Activity-dependent enhancement of synaptic transmission, referred to as long-term potentiation (LTP), is observed at many synapses in the central nervous system. In the hippocampus two distinct forms of LTP have been identified. One involves the activation of the NMDA (N-methyl-D-aspartate) subtype of glutamate receptor and a rise in postsynaptic Ca^{2+} , whereas the other, which is found at mossy fibre synapses, is independent of NMDA receptors but does require a rise in presynaptic Ca^{2+} . Although it is now generally accepted that mossy fibre LTP is expressed presynaptically, the locus of expression for NMDA-receptor-dependent LTP is controversial. Here the two forms of LTP are compared and it is argued that the balance of evidence favours a postsynaptic locus for NMDA-receptor-dependent LTP.

It is now generally accepted that long-lasting, activity-dependent changes in synaptic strength are of fundamental importance for the development of neural circuitry and for information storage in the mammalian brain. The first demonstration of such long-lasting changes in synaptic strength, elicited by brief repetitive stimulation, was at synapses made between perforant path fibres and granule cells in the dentate gyrus of the hippocampus¹. Subsequent studies established that a similar potentiation, referred to as long-term potentiation (LTP), could be elicited at the Schaffer collateral/commissural synapses in the CA1 region of the hippocampus² as well as at the mossy fibres synapses in the CA3 region of hippocampus³. Over the past decade, much has been learned about the properties and mechanisms of LTP at these synapses⁴⁻⁶. Thus these forms of LTP have served as prototypes to which other forms of synaptic plasticity observed in different brain regions, including the cerebral cortex, are compared.

All three types of excitatory synapses in the hippocampus use glutamate as their neurotransmitter. Glutamate is known to bind to both ionotropic and metabotropic receptors. The ionotropic receptors can be further subdivided into at least two types, termed α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and NMDA receptors (AMPA receptors and NMDARs, respectively), based on the selective glutamate agonists used to distinguish them. Although the LTP recorded at these three sites is superficially similar, it is well established that blockade of NMDARs blocks the induction of LTP in both the CA1 region and dentate gyrus⁴, but is without effect on mossy fibre LTP⁷. Recent evidence suggests that the difference between mossy fibre LTP and NMDAR-dependent LTP extends far beyond the role of the NMDAR. This progress article aims to summarize briefly the salient properties and issues of NMDAR-dependent LTP and then contrast these to mossy fibre LTP. Although a prominent feature of LTP is its extremely long duration, we will concern ourselves with changes that occur during the first hour of LTP, the period most thoroughly investigated. For more comprehensive discussion of NMDAR-dependent LTP, the reader is referred to some recent reviews⁴⁻⁶.

NMDA receptor-dependent LTP

Induction mechanisms. There is wide agreement that NMDAR-dependent LTP is triggered by the influx of Ca^{2+} through the NMDAR during the depolarization associated with repetitive synaptic activation. However, it is still not clear whether a rise in Ca^{2+} alone is a sufficient trigger^{4,5}. Additional factors may be required, one controversial candidate being activation of G-protein-coupled metabotropic glutamate receptors⁸.

Expression mechanisms. The sequence of events that occurs following the rise in Ca^{2+} remains controversial. It is generally thought that Ca^{2+} -dependent protein kinases are involved. Specifically it has been reported that inhibitors of protein kinase C⁹⁻¹¹ and Ca^{2+} /calmodulin-dependent protein kinase II (CAM kinase II)¹¹⁻¹³ can block LTP. In addition, transgenic mice lacking the α -subunit of CAM kinase II exhibit markedly impaired LTP¹⁴. In terms of what happens after kinase activation, opinion diverges drastically into presynaptic and postsynaptic camps. A relatively simple postsynaptic scenario involves the phosphorylation of the AMPAR by CAM kinase II, a major constituent of the postsynaptic density, resulting in an upregulation in AMPAR function. In this model, both the induction and expression of LTP would be localized within the postsynaptic spine. Indeed, elevation of postsynaptic Ca^{2+} either by activation of voltage sensitive Ca^{2+} channels¹⁵ or by activation of NMDARs¹⁶ in slices, but not in culture¹⁷, causes a large increase in the amplitude of miniature excitatory postsynaptic currents (e.p.s.cs) and enhances responses to the exogenously applied glutamate agonist AMPA. Tetanus-induced LTP is also reported to be associated with a delayed increase in AMPAR sensitivity¹⁸. These parameters are generally accepted as reflecting postsynaptic changes. The Ca^{2+} dependent enhancement of miniature e.p.s.cs is blocked by kinase inhibitors, in particular by KN-62, a selective blocker of CAM kinase II¹⁹.

It has been reported that activated CAM kinase II can phosphorylate AMPARs located in the postsynaptic density and when injected into cultured neurons enhances responses to the non-NMDAR agonist kainate²⁰. Furthermore, activation of NMDARs on cultured neurons results in the phosphorylation of AMPARs by CAM kinase II²¹. Finally, it has been reported that LTP is associated with an increase in Ca^{2+} -independent CAM kinase II activity²². These findings all support a simple postsynaptic model for LTP. However, the potentiation evoked by activation of voltage-sensitive Ca^{2+} channels²³⁻²⁵, and perhaps also by the application of NMDA^{26,27}, does not appear to be entirely equivalent to LTP; thus it will be important to determine more precisely the relationship between LTP and these other two forms of Ca^{2+} -dependent potentiation.

In what way might LTP, or more specifically CAM kinase II, upregulate AMPAR function? There is precedence for the phosphorylation-induced upregulation of non-NMDARs. Cyclic AMP is known to enhance kainate responses on retinal horizontal cells²⁸ and cultured hippocampal neurons^{29,30}. The molecular biology of these receptors is unknown, but it is well documented that the GluR6 subtype of receptor contains a protein kinase A consensus sequence and is upregulated by

cAMP^{31,32}. The enhancement of AMPAR function by phosphorylation could involve changes in single-channel properties or in the insertion and/or unmasking of clusters of functional AMPARs. The latter mechanisms would be consistent with the observed decreases in failures of synaptic transmission seen with minimal stimulation (see below) and with the reported increases in AMPAR binding sites following LTP³³.

Even if we accept that LTP involves a postsynaptic modification, this does not rule out presynaptic changes. Presynaptic modifications most often occur by changes in the probability that a synapse will release a quantum from a release site. A number of approaches have been used to address and support the hypothesis that LTP involves a presynaptic modification, and this has recently been reviewed extensively⁴. Perhaps the most suggestive evidence comes from studies employing minimal stimulation in which only one or a few fibres are stimulated. Under these conditions, stimulation often fails to elicit any postsynaptic response, and there is wide acceptance that shortly after the induction of LTP there is a decrease in the incidence of failures^{34–38}.

A decrease in the incidence of failures is routinely thought to indicate that the probability of neurotransmitter release (P_r) has increased. However, the decrease in the rate of failures during LTP could also be explained if there were an upregulation of AMPARs at synapses that before LTP were silent because they did not contain functional AMPARs³⁹. A comparison of the trial-to-trial fluctuation of AMPAR and NMDAR e.p.s.cs is consistent with the idea that there is a population of synapses which contain NMDARs but no functional AMPARs⁴⁰. A more direct examination of the existence of silent synapses has been accomplished by comparing the responses evoked by minimal stimulation at negative membrane potentials (AMPA only responses) and at positive potentials (AMPA and NMDAR responses)^{41,42}. Stimuli that evoke no detectable AMPAR e.p.s.c. at negative potentials can often evoke NMDAR e.p.s.cs at positive potentials. In addition, when stimulation of these silent synapses is paired with depolarization, AMPAR e.p.s.cs appear following the pairing. These findings provide a simple postsynaptic mechanism to account for the change in failures that is seen with LTP. Consistent with this hypothesis is the observation that LTP expression can occur primarily on the AMPAR component of the synaptic responses, whereas manipulations which change P_r cause an equal change in both the AMPAR and NMDAR component^{40,43–46}.

Although the above findings support a postsynaptic involvement in the expression of LTP, recent work in cultured hippocampal cells has reported an increase in synaptic vesicle turnover following NMDAR activation⁴⁷. In hippocampal slices, to address a presynaptic involvement in LTP⁴⁸, the irreversible use-dependent NMDAR antagonist MK-801 has been used to monitor small changes in P_r ^{49,50}. However, this technique failed to reveal any change in P_r during LTP⁴⁸, suggesting that changes in P_r may not contribute importantly to LTP in the slice preparation.

As has been emphasized repeatedly⁴, if there is a presynaptic component to the expression of LTP, a diffusible retrograde messenger must transmit the induction signal from the postsynaptic cell to the presynaptic terminal. Several candidate messengers have been investigated with the most prominent being nitric oxide (NO), which is known to be generated in a Ca^{2+} -dependent manner, following activation of NMDARs⁵¹. However it appears that NO is not absolutely required for LTP^{52–54}. This forces the conclusion that if a presynaptic modification contributes to LTP, there must be retrograde messengers in addition to NO. Evidence for the existence of a diffusible LTP signal that can be produced by the postsynaptic cell has recently been provided⁵⁵. Based on simultaneous paired recordings from pyramidal cells, it was reported that selectively inducing LTP in one of the cells resulted in the appearance of LTP in a nearby pyramidal cell. But, the action of this signal at recipient synapses is

dependent on postsynaptic Ca^{2+} in the neighbouring cell, thus the signal may not target presynaptic terminals.

It should be noted that prolonged 1–5 Hz afferent stimulation elicits a long-term depression (LTD) of synaptic strength in the CA1 region and can rapidly switch off the expression of LTP in an NMDAR-dependent manner⁸. This finding indicates that if a retrograde messenger is required for LTP, the maintenance of LTP must have at least one of the two following remarkable properties: either the 'depotentiating' stimulus must generate another NMDAR-dependent retrograde signal to turn off the presynaptic expression mechanism that was initiated by the original retrograde signal, or the maintenance of LTP must require the continued production and delivery of the messenger(s) from the postsynaptic cell. The moment-to-moment release of transmitter at a synapse would thereby be controlled by the constitutive release of retrograde message. The complexity associated with a presynaptic expression mechanism contrasts with the simplicity of a postsynaptic expression mechanism, in which each of the proposed steps involves more extensively studied molecular mechanisms. The morphological changes in dendritic spine structure associated with LTP, which may occur rapidly and almost certainly contribute to the later phase of LTP, are consistent with a postsynaptic mechanism, and in turn may initiate compensatory changes in presynaptic structures⁵⁶.

Mossy fibre LTP

Induction mechanisms. The first indication that mossy fibre synapses might have different functional properties from other excitatory synapses in the hippocampus came from the observation that, unlike most areas of the hippocampus, NMDAR binding was very low in stratum lucidum, the termination zone of mossy fibres⁵⁷. Soon afterwards it was found that NMDAR activation is not involved in the induction of LTP at this synapse^{7,58}. The lack of involvement of NMDARs in mossy fibre LTP could indicate a fundamentally different form of LTP. Alternatively, it could indicate that the rise in postsynaptic Ca^{2+} does not require NMDARs and instead occurs as a result of (1) voltage-dependent Ca^{2+} channel activation; (2) metabotropic glutamate receptor activation; or (3) Ca^{2+} entry through a Ca^{2+} permeable non-NMDAR. Initial experiments on this issue were controversial. Some evidence suggested that mossy fibre LTP was independent of postsynaptic Ca^{2+} and postsynaptic membrane potential^{58,59}, whereas other evidence indicated the opposite^{60–62}. Subsequent studies from independent laboratories confirmed the original results ruling out postsynaptic Ca^{2+} and membrane potential in the induction mechanism^{63,64}. More recently, the effect on mossy fibre LTP of blocking synaptic transmission postsynaptically with glutamate-receptor antagonists or presynaptically by removing extracellular Ca^{2+} has been examined^{65,66}. Postsynaptic blockade of mossy fibre synapses during a tetanus had no effect on the magnitude of LTP recorded following the washout of the glutamate receptor antagonists. In contrast, removal of extracellular Ca^{2+} to prevent any Ca^{2+} influx into the presynaptic terminal completely blocked the induction of LTP. These findings indicate that mossy fibre LTP is fundamentally different from NMDAR-dependent LTP. Its induction requires a rise in presynaptic Ca^{2+} and is independent of postsynaptic Ca^{2+} and postsynaptic membrane potential.

Expression mechanisms. The issue of whether the expression of mossy fibre LTP is pre- or postsynaptic has received less attention than LTP in the CA1 region. One approach has been to analyse the fluctuation underlying transmitter release, but this analysis has yielded different results depending on the assumptions used in the analysis^{67,68}. Another approach has been to examine for possible interactions between mossy fibre LTP and paired-pulse facilitation (PPF), a presynaptic phenomenon observed at most chemical synapses. Manipulations that affect transmitter release interact strongly with PPF⁶⁹, as does mossy fibre LTP^{58,68,70}. It has also been reported that the incidence of failures decreases with mossy fibre LTP⁶⁸. Recently, it has been

established that mossy fibre synapses activate NMDARs⁷¹. This finding has allowed the use of the MK-801 technique to address the site of expression of mossy fibre LTP. Contrary to the results in the CA1 region, a large increase in P_r during mossy fibre LTP was observed⁷¹.

What signalling pathway connects the transient rise in Ca^{2+} in the presynaptic terminal to the long lasting enhancement of evoked glutamate release? Recent evidence indicates that cAMP fulfills many of the requirements for the missing second messenger. Brief application of the adenylyl cyclase activator forskolin^{72,73} or membrane-permanent analogues of cAMP⁷³ causes a long-lasting presynaptic enhancement of mossy fibre responses that occludes LTP. In addition, a number of blockers of protein kinase A, including both catalytic and regulatory site blockers, block mossy fibre LTP^{73,74}. These results indicate that the increase in cAMP and activation of protein kinase A are obligatory in this form of synaptic plasticity, and that increases in presynaptic Ca^{2+} must be able to activate adenylyl cyclase. Cloning studies have in fact shown that there are a variety of isoforms of adenylyl cyclase, some of which are strongly activated by Ca^{2+} /calmodulin (for example, type I)⁷⁵. The type I isoform has been shown to be expressed in uniquely high amounts in dentate granule cells⁷⁶, which give rise to the mossy fibres. Thus the rise in Ca^{2+} can, via calmodulin, directly activate the adenylyl cyclase.

Perhaps the most interesting question is how does activation of protein kinase A enhance release? One possibility is modulation of presynaptic Ca^{2+} channels. Pharmacological evidence⁶⁶ suggests that glutamate release from mossy fibres involves both N- and P-type calcium channels. When either N- or P-type channels are completely blocked during the tetanus, LTP of normal magnitude could be evoked, indicating that the Ca^{2+} required for inducing LTP can enter through either of these channel types.

Furthermore, the relative contribution of N- and P-type Ca^{2+} channels to synaptic transmission does not change during LTP, suggesting that LTP either upregulates Ca^{2+} entry through both types of channel to the same extent, or the enhancement occurs at some step after Ca^{2+} entry. This latter possibility is particularly intriguing in that the cAMP cascade would modify some component of synaptic vesicle release machinery.

Conclusions

We have focused our attention on LTP found in the hippocampus. This structure contains synapses that exhibit two very distinct forms of synaptic plasticity. These differences are summarized in Fig. 1. NMDAR-dependent LTP is found at a number of excitatory synapses in the hippocampus, including the CA1 region, and at a variety of synapses throughout the brain. This form of LTP is induced by a transient rise in postsynaptic Ca^{2+} , and there is evidence that it involves Ca^{2+} -dependent kinases, in particular CAM kinase II. Whether the expression of this form of LTP involves a postsynaptic enhancement of AMPAR function and/or enhancement of transmitter release, remains the most hotly debated issue in this field. A postsynaptic expression mechanism certainly has the advantage of simplicity and can account for most of the experimental findings, but we must await more definitive experiments to know whether nature has opted for simplicity or for a more complex scheme.

Mossy fibres, which arise from dentate granule cells and synapse on the proximal dendrites of CA3 pyramidal cells, exhibit a form of LTP that is fundamentally different from NMDAR-dependent LTP. Recent evidence suggests that the postsynaptic cell plays no role, either in the induction or the expression of this form of LTP. Mossy fibre LTP is, however, dependent upon Ca^{2+} entry, but in this case into the presynaptic terminal. Phar-

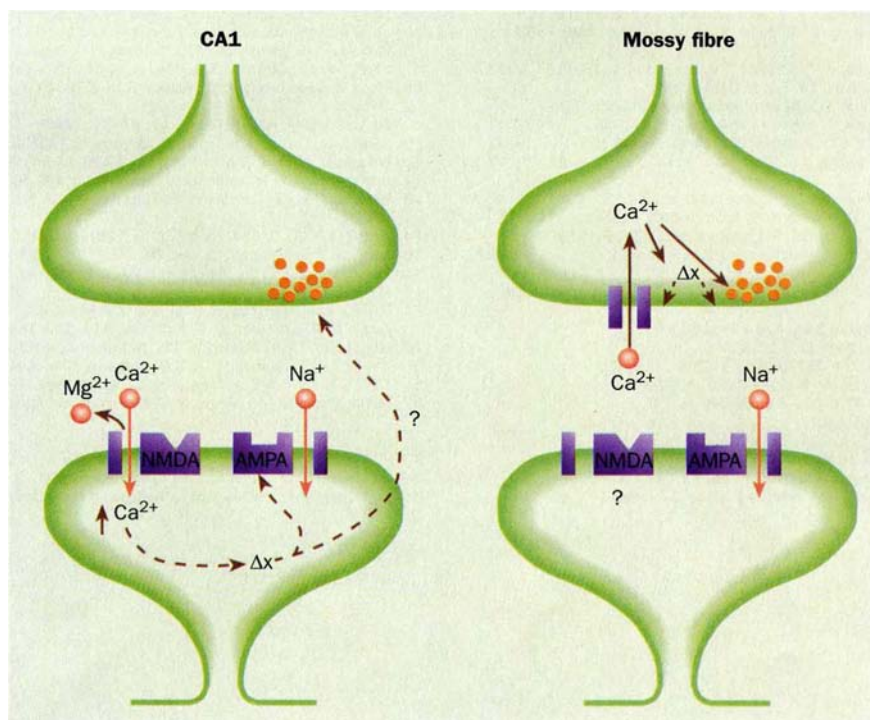


FIG. 1 Summary diagram of the two forms of LTP found in hippocampus. The LTP found in the CA1 region is dependent on activation of NMDA receptors and a rise in postsynaptic Ca^{2+} . Activation of a Ca^{2+} -dependent process (ΔX), most likely involving Ca^{2+} /calmodulin-dependent kinase II, is postulated to upregulate AMPA receptors, but the generation of a retrograde signal back to the presynaptic terminal may also

occur. Although NMDA receptors are present at mossy fibre synapses, the LTP at this synapse is independent of NMDA receptors, the functions of which are unclear at this synapse. Mossy fibre LTP is dependent on a rise in presynaptic Ca^{2+} , which activates a process (ΔX), most probably involving a Ca^{2+} /calmodulin-regulated adenylyl cyclase, that results in a persistent enhancement in evoked transmitter release.

macological studies suggest a simple model in which Ca^{2+} /calmodulin-stimulated adenylyl cyclase I is activated by Ca^{2+} entry during the tetanus used to generate LTP. This results in a transient rise in cAMP which activates protein kinase A. The mechanism by which protein kinase A enhances transmitter release remains to be determined.

Synaptic plasticity at a variety of peripheral sympathetic synapses^{77,78}, as well as at a variety of invertebrate synapses^{79,80}, share a number of features with mossy fibre LTP, including a presynaptic induction and expression mechanism and a role for cAMP. Curiously, although NMDAR-dependent LTP is found throughout the brain, an LTP with properties similar to mossy fibre LTP has not yet been reported. One might predict that this form of plasticity would be limited to those synapses that contain high levels of Ca^{2+} /calmodulin-stimulated adenylyl cyclase in the presynaptic terminal. The cellular properties that characterize these two forms of LTP indicate that NMDAR-dependent

LTP will occur only at those synapses that are active when the postsynaptic cell is depolarized by other inputs, whereas for mossy fibre LTP, all synapses made by a single granule cell will undergo LTP following repetitive activation of that granule cell. Thus, it is likely that these two forms of LTP subserve different functions as information is processed and stored within the hippocampus. One hypothesis is that mossy fibres are critical for the processing of information which is then presented to an autoassociative network that stores new information via Hebbian/NMDAR-dependent synaptic plasticity^{81,82}. As we learn more about the mechanisms of these two forms of LTP, their role in normal and pathological hippocampal function should become more apparent. □

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ACKNOWLEDGEMENTS. R.A.N. is a member of the Keck Center for Integrative Neuroscience and the Silvio Conte Center for Neuroscience Research. R.C.M. is a member of the Center for Neurobiology and Psychiatry. Both investigators are supported by grants from the NIH.

Crystal structure of a TFIIB–TBP–TATA-element ternary complex

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The crystal structure of the transcription factor IIB (TFIIB)/TATA box-binding protein (TBP)/TATA-element ternary complex is described at 2.7 Å resolution. Core TFIIB resembles cyclin A, and recognizes the preformed TBP–DNA complex through protein–protein and protein–DNA interactions. The amino-terminal domain of core TFIIB forms the downstream surface of the ternary complex, where it could fix the transcription start site. The remaining surfaces of TBP and the TFIIB can interact with TBP-associated factors, other class II initiation factors, and transcriptional activators and coactivators.

THE molecular bases of transcription initiation and its regulation are best understood for genes transcribed by RNA polymerase II (pol II) with the general initiation factors TFIIA, -B, -D, -E, -F, -G/J, -H and -I (reviewed in refs 1–3). This process can begin with TBP-mediated recognition of a TATA element by TFIID, forming a multiprotein–DNA complex that coordinates accretion of class II initiation factors and pol II into a preinitiation complex (PIC) (reviewed in ref. 4). Although incapable of substituting for native TFIID in assays of activator-dependent transcription, recombinant TBP alone can bind both general and regulatory factors (reviewed in ref. 5) and direct PIC assembly and basal (core promoter) transcription. TFIIB is the second general transcription factor to enter the PIC, creating a TFIIB–TFIID(TBP)–DNA platform that is in turn recognized by a complex of pol II and TFIIF (pol/F). *In vitro* studies with negatively supercoiled templates demonstrated that transcription initiation can be reconstituted with TBP, TFIIB and pol II, indicating that together TBP and TFIIB could position pol II (ref. 6). Mutants of TFIIB alter pol II start sites in yeast, providing compelling evidence for its function as a precise spacer or bridge between TBP and pol II on the core promoter that determines the transcription start site. TFIIB also interacts with *Drosophila* TAF_{II}40 (TAF for TBP-associated factor) and its human homologue, the general initiation factor TFIIF, and some transcriptional activators and coactivators (reviewed in refs 7, 8).

In vivo and under different conditions *in vitro*, pol II transcription initiation depends on TFIIE, TFIIF and TFIIH, and possibly TFIIA. Once PIC assembly is complete and in the presence of nucleoside triphosphates, strand separation at the transcription site occurs to give an open complex, the carboxy-terminal domain of the large subunit of pol II is phosphorylated, and pol II initiates transcription and is released from the promoter. During elongation, TFIID can remain bound to the core promoter supporting rapid reinitiation of transcription by pol II and the other general factors (reviewed in ref. 9). Core-promoter binding by the TBP subunit of TFIID is an intrinsically slow step¹⁰ because of the marked DNA deformation induced in the TATA element (reviewed in ref. 11). An abbreviated PIC assembly mechanism has also been proposed, following recent discov-

eries of various pol II holoenzymes containing many, if not all, of the general initiation factors except for TFIID (reviewed in ref. 12).

TFIIB's critical role has made it the focus of considerable biochemical and genetic study. Publication of the sequence of human TFIIB was followed rapidly by the sequences of homologous genes from a variety of eukaryotes and two archaeobacteria (amino-acid identities range from 99 to 32%; reviewed in ref. 13). The proteolytically stable C-terminal/core region consists of two 84-amino-acid direct repeats (20% sequence identity), binds to the TFIID(TBP)–TATA-element complex with high affinity but not to TFIID(TBP) alone^{7,14–18}, interacts with the C-terminal stirrup of TBP (ref. 19) and DNA upstream and downstream of the TATA box¹⁸. The N-terminal portion of TFIIB contains a putative Zn²⁺-binding sequence CysX₂CysX₁₇CysX₂Cys, and is essential for assembly of pol/F into the PIC^{7,14–18}.

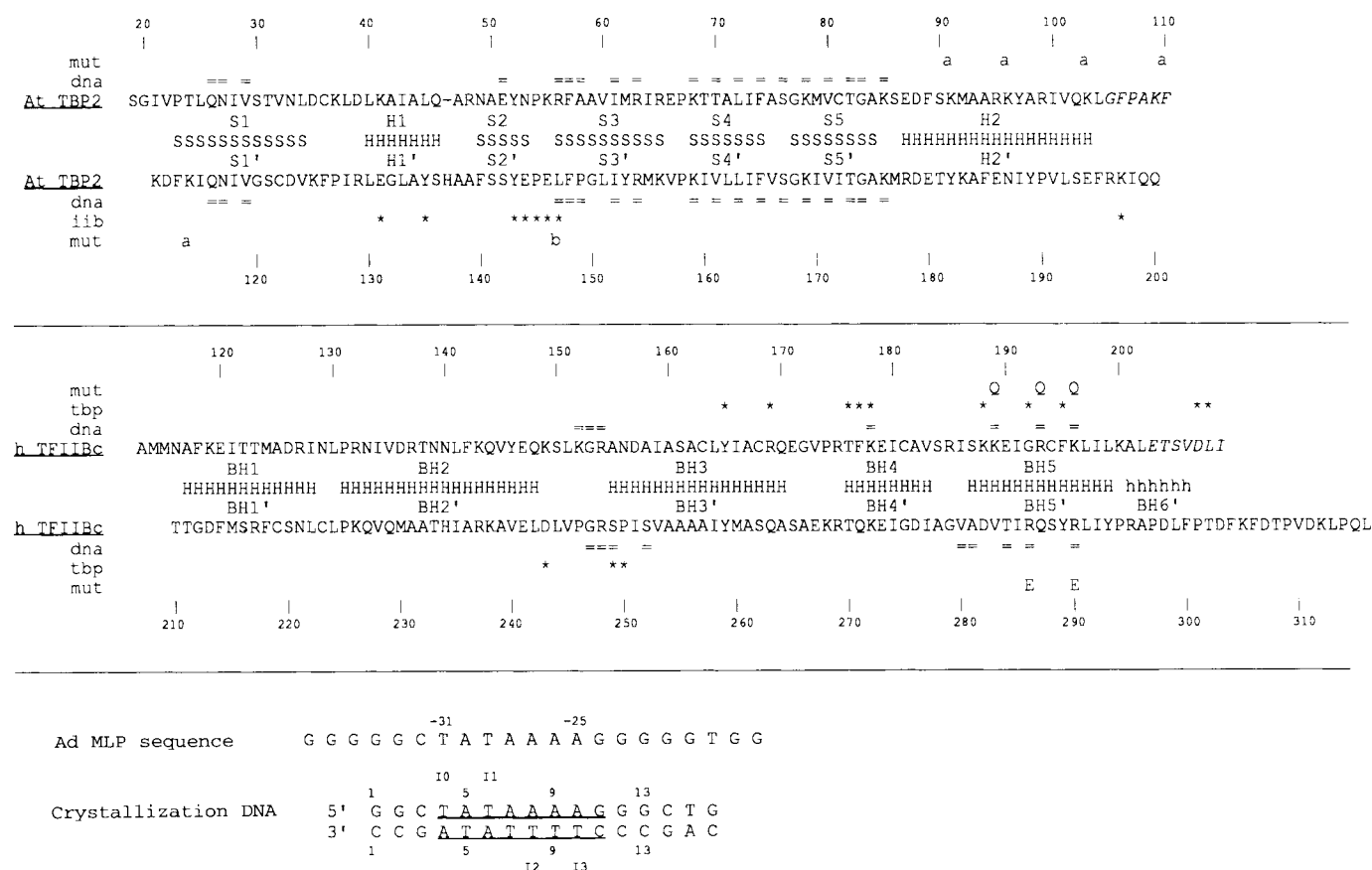
Recently we reported the X-ray crystal structures of TBP isoform 2 (TBP2) from *Arabidopsis thaliana*^{5,20} and its complex with the TATA element of the adenovirus major late promoter (AdMLP)^{11,21}. TBP displays a novel, two-domain DNA-binding fold, and the protein's approximate two-fold intramolecular symmetry generates a saddle-shaped structure dominated by a curved antiparallel β-sheet, forming a concave DNA-binding surface. TATA element recognition proceeds through an induced-fit mechanism, involving subtle conformational changes at the level of the protein and an unprecedented DNA distortion. The seat of the saddle presents a large convex surface to which other proteins bind during initiation of transcription. The structures of TBP2 and its complex with the AdMLP demonstrated that it could bind both DNA and a myriad of other transcription factors, but did not explain specific recognition by TFIIB and other components of the pol II transcription machinery.

We now report the crystal structure of a ternary complex of human transcription factor IIB, bound to TBP2 from *Arabidopsis thaliana*, which is in turn recognizing the TATA element of the AdMLP. The 2.7-Å resolution structure reveals that core TFIIB (cTFIIB) is a two-domain α-helical protein that resembles cyclin A and interacts with the C-terminal stirrup of TBP, making contacts with the phosphoribose backbone of the core promoter both upstream and downstream of the centre of the TATA element. This structure clarifies the nature of the interactions that permit TFIIB to recognize the TBP–DNA complex, provid-

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ing a stable platform for PIC assembly and interactions with transcriptional activators and coactivators.

Structure determination and refinement

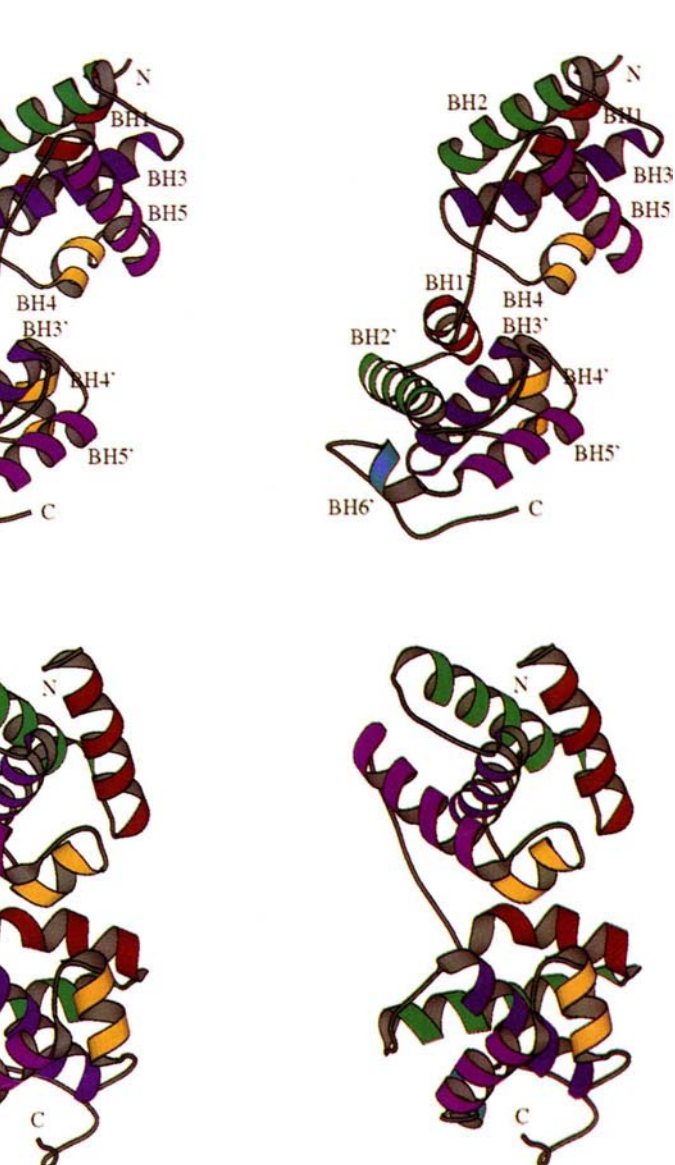
Human cTFIIB was expressed, purified and cocrystallized with *Arabidopsis thaliana* TBP2 complexed with a 16-base-pair (bp) oligonucleotide bearing the AdMLP TATA element (Fig. 1a, d, e; Table 1). Cocrystals grow in the orthorhombic space group $P2_12_12_1$ ($a = 69.9$ Å, $b = 78.0$ Å, $c = 134.7$ Å) with one ternary complex in the asymmetric unit, and diffract anisotropically to at least 2.3 Å. The X-ray structure was determined at 2.7-Å resolution by a combination of molecular replacement and multiple isomorphous replacement (MIR) (Fig. 1; Table 1), followed by model building and X-PLOR refinement²² (Fig. 1d, e; Table 1). The current crystallographic model consists of three components: the 16-bp oligonucleotide, TBP2(11–198) and TFIIB(112–316). The electron density for the polypeptide backbone is everywhere continuous at 1.2σ in a $(2|F_{\text{observed}}| - |F_{\text{calculated}}|)$ difference Fourier synthesis, except for a break in the loop connecting helices BH2' and BH3' (Fig. 2). PROCHECK²³ revealed 7/393 unfavourable backbone torsion angle combinations, and main-chain and side-chain structural parameters consistently better than those expected at 2.7-Å resolution (overall G -factor = 0.0). The DNA electron density is well defined throughout. With the exception of three well-ordered water molecules located within cavities in the interior of TBP2, no solvent molecules have been included at this stage in the refinement. A full account of the refinement at the diffraction limit will be published elsewhere.

Core TFIIB structure

TFIIB(112–316) is illustrated in Fig. 1a–c. The molecule has dimensions $65 \text{ Å} \times 32 \text{ Å} \times 32 \text{ Å}$, with two similar domains rotated by 90° with respect to one another and connected by a short linker (domain radius = 16 Å). There is no simple two-fold rotation relating the two domains as in TBP2 (ref. 20). Each domain (amino-acid identity = $17/84 = 20\%$) comprises five α -helices (designated BH1–BH5 and BH1'–BH5'), arranged in a compact globular domain with BH3(') in the hydrophobic core. Beyond BH5' is a short α -helix (BH6') and a random-coil segment extending to the molecule's C terminus. A cleft separates the domains, which are connected in the amino-acid sequence by a short random-coil interdomain peptide (Glu 203 to Ile 209). There are limited interdomain hydrophobic contacts (total buried surface area = 600 Å^2), between residues in BH4 and the BH4'–BH5' loop and between residues in BH1' and the BH3–BH4 loop, making some interdomain motion possible. The N terminus of TFIIB was not examined in this study.

Threading calculations²⁴ confirmed the compatibility of the structure of human cTFIIB with the amino-acid sequences of all known TFIIBs (z scores = 40–15). Our structure is also compatible with the N-terminal sequence of yeast PCF4/TDS4/BRF1 and human TFIIB90, pol III transcription factor homologues of TFIIB (refs 25–28) (z score = 14). Thus it may be possible to build a homology model of the TFIIB-like region of yeast PCF4/TDS4/BRF1 and human TFIIB90 from our structure of cTFIIB and to develop testable hypotheses concerning their roles in pol III transcription. As predicted by Kouzar-

FIG. 1 a, Amino-acid sequences of TBP2 from *Arabidopsis thaliana* and human cTFIIB, each with their two structural repeats aligned one above the other. Residues in α -helices and β -strands are denoted by H and S, respectively, and the secondary structural features are labelled as discussed in the text (interdomain peptide residues are shown in italics, and h denotes BH6' in the second domain of cTFIIB). For TFIIB the human numbering scheme is used throughout, and for TBP the TBP2 numbering scheme is used throughout (to convert to human and *S. cerevisiae* add 136 and 42, respectively). The locations of residues in the ternary-complex crystal structure making DNA contacts are denoted by equals signs, and contacts between TBP2 and cTFIIB by asterisks. The locations of point mutants and their phenotype are indicated as: a, TFIIB binding (reviewed in ref. 5); b, TFIIB binding¹⁹; Q, TBP binding when all are changed to Gln (ref. 17), and E, TBP binding when all are changed to Glu (ref. 7). Partial sequence of the AdMLP aligned with the sequence of the duplex oligonucleotide employed in cocrystallization, showing the locations of the iodine-labelled bases used for the MIR structure determination. The numbering scheme of the AdMLP is used throughout the text. The 8 base pairs involved in TBP-DNA recognition are underlined. b, c, Structure of human cTFIIB. The N and C termini of the protein are labelled, and the α -helices of each domain are numbered (BH) and coloured in order red, green, blue, yellow and magenta. The primes refer to the second structural domain, and the BH6' helix absent from the first domain is coloured light blue. b, Upper pair, stereo drawing⁴⁷ of a ribbon representation of the three-dimensional structure viewed down the cleft between the two quasi-identical domains; lower pair, stereo drawing viewed perpendicular to the cleft. c, Upper pair, stereo-drawing superimposition of the α -carbon backbone of the first (red) and second (green) domains comprising cTFIIB. α -Carbon atomic positions of residues 117–199 and 211–293 were used to determine the least-squares superimposition of the two domains (r.m.s.d. = 1.1 Å for α -carbon atomic pairs). Lower pair, stereo-drawing superimposition of the α -carbon backbone of the first domains of cTFIIB (red) and cyclin A (light blue). R.m.s.d. = 1.5 Å for α -carbon atomic pairs in this superimposition (14/93 = 15% sequence identity), and 1.7 Å for the corresponding superimposition of the second domains (9/90 = 10% sequence identity). cTFIIB and cyclin A cannot be superimposed in their entirety because the relative dispositions of the two domains are completely different. d, TBP2 reconstitutes the transcriptional activity of heat-treated nuclear extract from HeLa cells with the same efficiency as hTBP. Specific transcription reaction from AdMLP in HeLa nuclear extract (lane 1), using the G-free cassette template. Inactivation of the reaction after heat treatment at 47 °C (lane 2). TBP2 (lanes 3 and 4) reconstitutes the transcriptional activity as efficiently as human TBP (lanes 5 and 6). e, TBP2 binds to the TATA-cocrystallization oligonucleotide and it forms a stable, specific complex with human cTFIIB in gel-mobility shift assays. 1 μ mol of labelled cocrystallization DNA (a) was incubated with either 0.8 μ mol of TBP2 alone (lanes 2, 3 and 4) or a cTFIIB-TBP2 mixture (lanes 5, 6 and 7). Lane 1 represents the migration of the free probe. Lanes 3 and 6 also include 10 μ mol of unlabelled heterologous oligonucleotide, and lanes 4 and 7 also include 10 μ mol of unlabelled cocrystallization DNA. Lane 8 shows cTFIIB without TBP2. METHODS. Human TFIIB residues 113–316, fused with an additional 20 N-terminal amino acids (MGSSHHHHHSSGLVPRGSH),



was overexpressed in *Escherichia coli* (BL21(DE3)pLysS) using the T7 RNA polymerase system⁴⁸. Cells grown at 30 °C to an absorbance of 0.7 at 595 nm and induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside for 3 h were collected by low-speed centrifugation. Lysis was performed by 3 cycles of freeze-thaw and DNA digestion was performed by DNase I. The soluble fraction was applied directly to a Ni²⁺-ion affinity column and washed with a buffer containing increasing amounts of imidazole. TFIIB fusion protein with an estimated homogeneity of 95% was then eluted from the resin with a buffer containing 100 mM EDTA. After dialysis overnight, removal of the histidine-containing N-terminal sequence was performed by trypsin proteolytic cleavage of the peptide bond between R and G in the above sequence to yield TFIIB(113–316) plus 4 N-terminal amino acids, namely GSHM. A final cation-exchange chromatography step to remove the protease and any remaining contaminants yielded material of homogeneity estimated to be 98% or greater. Mass spectrometry documented that the TFIIB(113–316) used for crystallization was neither modified nor further proteolysed during expression and purification. The measured molecular mass was 22,934 $\pm$ 3, compared with 22,931 calculated from the predicted amino-acid sequence. Dynamic light scattering (Molecular Size Detector, Protein Solutions, Charlottesville, VA) showed that TFIIB(113–316) is both monomeric and monodisperse at 1 mg ml⁻¹. TBP2 and the cocrystallization DNA were prepared as described previously^{20,21}.

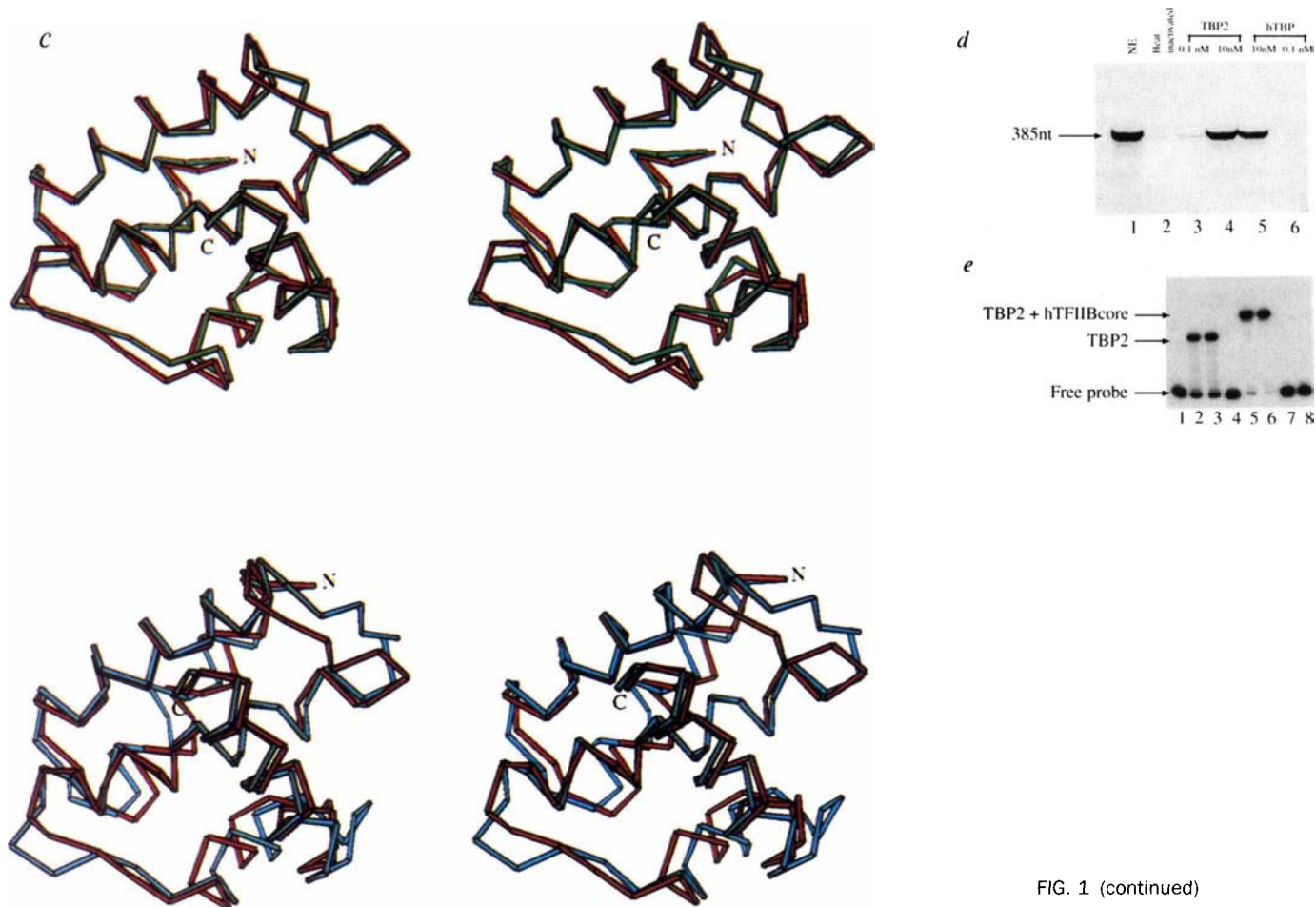


FIG. 1 (continued)

ides *et al.*²⁹, cTFIIIB resembles cyclin A (ref. 30; L. N. Johnson *et al.*, manuscript in preparation). Although each domain of cyclin A can be superimposed on the corresponding domain of cTFIIIB (Fig. 1), the relationship between the two domains is different, as are the interdomain contacts.

TFIIIB-TBP-DNA ternary complex structure

Overview. Figure 2 illustrates the structure of the cTFIIIB-TBP2-DNA ternary complex. cTFIIIB clamps the acidic C-terminal stirrup of TBP2 (S2'-S3') in its cleft and also interacts with H1', the C terminus (Figs 1 and 2), and the phosphoribose backbone upstream and downstream of the centre of the TATA element (Figs 2-5). Although the two domains of cTFIIIB have the same fold, they do not have chemically identical surfaces (Fig. 5) and cannot make equivalent interactions with TBP2. Contacts between cTFIIIB and the C-terminal stirrup of TBP2 are made by BH3, BH4 and BH5. The interdomain peptide interacts with H1' and the C-terminal stirrup of TBP2. cTFIIIB's BH2'-BH3' loop interacts with the same stirrup and the C terminus of TBP2. Ternary complex formation buries about 5,600 Å² of solvent-accessible surface area. The TBP2-TATA-element binary complex buries about 2,900 Å². Addition of cTFIIIB results in the occlusion of another 700 Å² of TBP2 surface and another 700 Å² of DNA surface, burying about 1,200 Å² of cTFIIIB surface in the process. These values are typical for protein-DNA and protein-protein molecular recognition events (reviewed in ref. 31), permitting the ternary complex to display extensive solvent-accessible surfaces for both TBP2 and cTFIIIB (about 7,900 Å² and 8,300 Å², respectively).

Core promoter DNA conformation in the ternary complex.

Figures 2 and 3 illustrate the markedly distorted structure of the oligonucleotide in the ternary complex. The right-handed double helix makes standard Watson-Crick base pairs throughout, with

bases in the *anti* conformation. Sugar puckers were restrained towards C2'-endo during crystallographic refinement, but they show no clear preference for any pucker at this resolution limit. Base-pair hydrogen-bonding restraints were also used during refinement.

A detailed analysis of the DNA conformation is presented in Fig. 4 with the corresponding values from our 1.9-Å resolution refinement of the TBP2-DNA cocrystal structure¹¹. Throughout the oligonucleotide, shift, slide and tilt values show minimal variation about zero. The first four and last six base pairs are essentially B-form ($\langle \text{rise} \rangle = 3.3$ Å, $\langle |\text{roll}| \rangle = 2.4^\circ$, $\langle \text{twist} \rangle = 35.2^\circ$ or 10.2 bp per turn). The intervening six base pairs (ATAAAA) demonstrate the same novel, partly unwound, right-handed double helix seen in cocrystal structures of TBP2 (refs 11, 21) and yeast TBP (ref. 32). The root-mean-square deviation (r.m.s.d.) between the 8-bp TATA elements in our binary and ternary complex structures is 0.6 Å for all non-hydrogen atoms and 0.4 Å for C1' atoms. Inclusion of the flanking base pairs common to the two structures gives r.m.s.d. = 0.9 Å for all non-hydrogen atoms. At the 5' end of the TATA element where the first phenylalanine-induced kink occurs, the T-A(-31):A-T(-30) step has rise = 5.0 Å, roll = 40° and twist = 19°. A similar situation prevails at the second phenylalanine-induced kink, where the A-T(-25):G-C(-24) step has rise = 5.4 Å, roll = 30° and twist = 26°. The 5' kink in the ternary complex is very similar to the corresponding kink in the TBP2-DNA complex, but the 3' kink in the ternary complex has a roll angle of only 30°, as against 45° in the TBP2-DNA complex. Despite these differences, the path of the distorted double helix in the ternary complex structure also involves a lateral displacement of 18 Å and a 110° angle between the cylindrical axes of incoming and outgoing B-form double helices (Fig. 3).

TABLE 1 Summary of crystallographic analysis

	Native	I-dU-02	I-dU-03	I-dU-12	I-dU-13
Resolution (Å)	2.7	2.6	2.7	2.6	2.9
Reflections (observations/unique)	180,515/20,995	171,615/23,514	179,390/20,995	170,701/23,459	139,467/17,127
Data coverage (%)	82.1	83.3	84.0	79.0	91.0
R_{sym} (%)	7.2	7.5	7.4	5.6	7.1
Mean fractional isomorphous difference (%)		12.5	12.5	11.5	13.5
MIR* analysis (18.0–2.7 Å)					
Phasing power		1.49	1.61	1.66	1.42
R_c		0.57	0.52	0.58	0.59
Mean overall figure of merit	0.74				
Refinement					
Resolution (Å)	8.0–2.7				
R factor/free R factor (%)	21.5/31.8				
Reflections ($ F > 1.5\sigma F $)					
working/test	14,676/1,619				
Total number of atoms	3,729				
R.m.s. bond length (Å)	0.014				
R.m.s. bond angle (deg)	1.86				
R.m.s. B-factor bonded atoms (Å ²)	1.86				

cTFIIB–TBP2–DNA complex was prepared to a final concentration of 0.3–0.4 mM in 40 mM KCl, 300 mM ammonium acetate, 5 mM MgCl₂, 5 mM CaCl₂, 10 μM zinc acetate, 10 mM dithiothreitol, 10% (v/v) glycerol, 2% (v/v) ethylene glycol, 40 mM Tris–HCl, pH 8.5. The ternary complex was relatively insoluble under low salt conditions, enabling crystals to be grown at 4 °C by vapour diffusion against a reservoir containing 5–10% (v/v) glycerol, 20 mM dithiothreitol, 40 mM Tris–HCl, pH 8.5. Chunky plates grew over the course of days with typical dimensions of 0.5 × 0.2 × 0.05 mm³. Crystals of heavy-atom derivatives were prepared in the same manner with DNA in which 5-iodouracil had been substituted for T in the positions denoted in Fig. 1a. Diffraction measurements were carried out at –150 °C using a Rigaku RAXIS-IIc imaging plate area detector and a refrigerated nitrogen gas delivery system. Crystals were transiently ‘cryoprotected’ in a mixture containing 10% (v/v) glycerol, 25% (v/v) ethylene glycol, 1 mM MgCl₂ and 5 mM Tris–HCl, pH 8.5, before freezing on a platform created by a loop of ophthalmological suture material (10-0 Ethilon, Ethicon). Oscillation photographs were integrated, scaled and merged with DENZO and SCALEPAK (Z. Otwinowski, personal communication). The 2.7-Å resolution native data used for structure refinement were 82% complete with $R_{\text{merge}}(I) = 7.2\%$ and $\langle I/\sigma(I) \rangle = 24$ (between 2.82 and 2.7 Å the data were 57% complete with $R_{\text{merge}}(I) = 19.7\%$ and $\langle I/\sigma(I) \rangle = 6.5$). Molecular replacement (MR) was carried out with X-PLOR²² using the structure of the TBP2–DNA complex¹¹. Patterson-correlation refinement of the rotation function search maxima yielded a solution with correlation coefficient 0.139. A translation search in X–Y–Z gave a final MR solution 14.5σ above the mean peak level. Rigid-body refinement of the two domains of the polypeptide chain and the two DNA strands in the asymmetric unit, followed by positional and simulated annealing refinement with X-PLOR, gave an R-factor of 0.37 at 2.7 Å resolution. Although the electron density for the TBP2–DNA complex was well defined in $(2|F_{\text{observed}}| - |F_{\text{calculated}}|)$ difference Fourier syntheses, there was no interpretable electron density for cTFIIB, necessitating the collection of X-ray diffraction data from four doubly iodinated DNA cocrystals (Fig. 1a). Iodine atoms were located by isomorphous difference Patterson analyses, and confirmed by isomorphous difference Fourier synthesis with phases from the refined MR solution. Refinement of the heavy atom model against both isomorphous and anomalous differences gave a final MIR figure of merit of 0.74 (ref. 46). The MIR density showed continuous electron density for almost the entire ternary complex. Knowledge of the mercury atomic positions obtained from a non-isomorphous heavy-atom derivative helped to fix the registration of TFIIB(113–316) in the complex (results not shown). Model-building interspersed with positional refinement allowed an unambiguous trace and sequence assignment of TFIIB(113–316). Atomic coordinates and structure factor amplitudes will be submitted to the Brookhaven Protein Data Bank. $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where I is observed intensity, $\langle I \rangle$ is average intensity obtained from multiple observations of symmetry-related reflections. Mean fractional isomorphous difference = $\sum ||F_{\text{PH}}| - |F_P|| / \sum |F_P|$, where $|F_P|$ is protein structure factor amplitude and $|F_{\text{PH}}|$ is heavy-atom derivative structure-factor amplitude. Phasing power = $\text{r.m.s.}(|F_{\text{H}}|/E)$, where $|F_{\text{H}}|$ is heavy-atom structure-factor amplitude and E is residual lack of closure. R.m.s. bond lengths and r.m.s. bond angles are the respective r.m.s. deviations from ideal values. R.m.s. thermal parameter is the r.m.s. deviation between the B values of covalently bonded atomic pairs.

* Multiple isomorphous replacement.

TBP2 structure in the ternary complex. The individual domains of TBP2 in the cTFIIB–TBP2–DNA ternary complex are identical to those in the TBP2–DNA complex^{11,21}, and in apo-TBP2 (refs 5, 20) (r.m.s.ds between α-carbon atomic positions are 0.4–0.6 Å). But ternary complex formation causes a small conformational change in TBP2, produced by a twisting motion of one domain relative to the other (a feature that also distinguishes the bound and apo structures). In this complex, the rotational displacement of the two domains is intermediate between DNA-bound and apo-TBP2 (results not shown). Presumably this modest structural perturbation reflects the differences seen in the TATA element, and results from interactions between cTFIIB and DNA (see below).

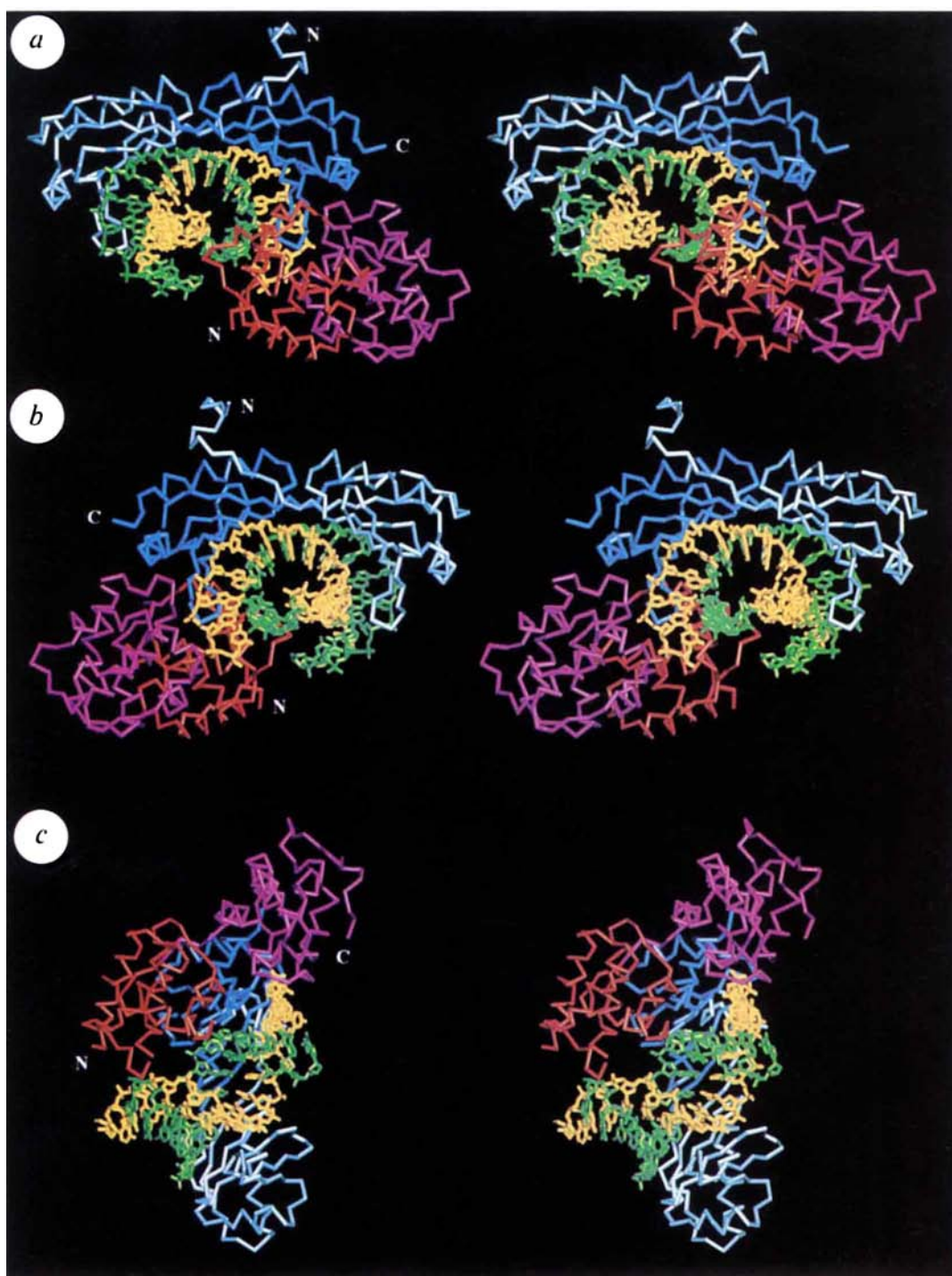
TATA element recognition by TBP in the ternary complex. Figure 4 depicts the TBP2–DNA contacts in the cTFIIB–TBP2–DNA ternary complex, which are essentially identical to those seen in our TBP2–DNA cocrystal structure^{11,21}. Once again, TBP2 recognizes the minor groove of the AdMLP TATA element via an induced-fit mechanism. Conformational changes in both the protein and the DNA generate complementary interaction surfaces without bad steric contacts between DNA and protein atoms. At this stage we do not see the water-mediated contacts revealed at 1.9 Å resolution¹¹. But we presume that

many of these water bridges will become apparent at higher resolution, and these putative interactions have been included in Fig. 4.

TBP–TATA element complex recognition by TFIIB. The structure of the cTFIIB–TBP2–DNA ternary complex represents the first example of a protein recognizing a preformed protein–DNA complex. Figures 2–5 illustrate features of the interactions that permit cTFIIB to recognize TBP2, which is in turn recognizing the TATA element. cTFIIB acts as a clamp, binding TBP2 in its cleft and interacting with the backbones of the coding and non-coding DNA strands upstream and downstream of the centre of the TATA element. There are no contacts between cTFIIB and major or minor groove edges of bases within the AdMLP, effectively precluding sequence-specific DNA binding.

Before describing this unique molecular recognition event in detail, the implications of cocrystallizing plant TBP2 with human cTFIIB must be addressed. Three lines of evidence document that this mixed-species structure is relevant to pol II transcription. First, control experiments demonstrated that TBP2 substitutes for human TBP in basal transcription, using heat-inactivated HeLa nuclear extract (Fig. 1d). Second, TBP2 forms a stable ternary complex with human cTFIIB in a gel mobility shift assay (Fig. 1e). Last, the 8 residues of TBP2 that interact

FIG. 2 TFIIB-TBP-DNA ternary complex. *a*, Stereo drawing viewed at 90° to TBP2's axis of approximate intramolecular two-fold symmetry from downstream, showing the -34 end of the AdMLP sequence entering the underside of the molecular saddle from below. The 3' end of the oligonucleotide exits the saddle as B-form DNA with its cylindrical axis nearly perpendicular to the page. cTFIIB grasps the TBP's second stirrup and interacts with the phosphodiester backbone upstream and downstream of the centre of the TATA element. Proteins are depicted as α -carbon traces and the DNA is drawn as an atomic stick figure. Molecules are colour coded as follows: cTFIIB first repeat, red; cTFIIB second repeat, magenta; TBP2 N terminus and first repeat, light blue; TBP2 second repeat, dark blue; DNA coding strand, green; DNA non-coding strand, yellow. N and C termini of TBP2 and cTFIIB are labelled when visible. The same colour coding scheme is used in Figs 3 and 5. *b*, Stereo drawing viewed perpendicular to TBP2's axis of approximate intramolecular two-fold symmetry from upstream of the TATA element. *c*, Stereo drawing viewed along TBP2's axis of approximate intramolecular two-fold symmetry from below the molecular saddle. *d*, Stereo drawing of the ternary complex interface showing cTFIIB interacting with both TBP2 and the DNA. Key residues in the intermolecular interface are labelled with the single-letter amino-acid code, and some hydrogen bonds and salt bridges are indicated with broken lines. *e*, Stereo drawing of the solvent-flattened MIR electron density (contour level 1.6 σ) for the portion of the ternary complex depicted in *d*, with the refined atomic model drawn as a stick figure.

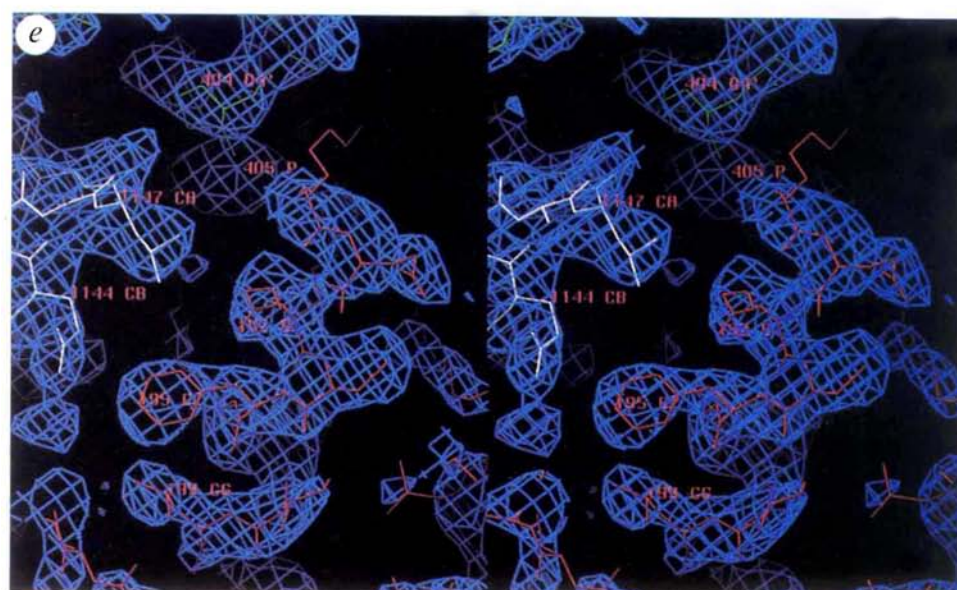
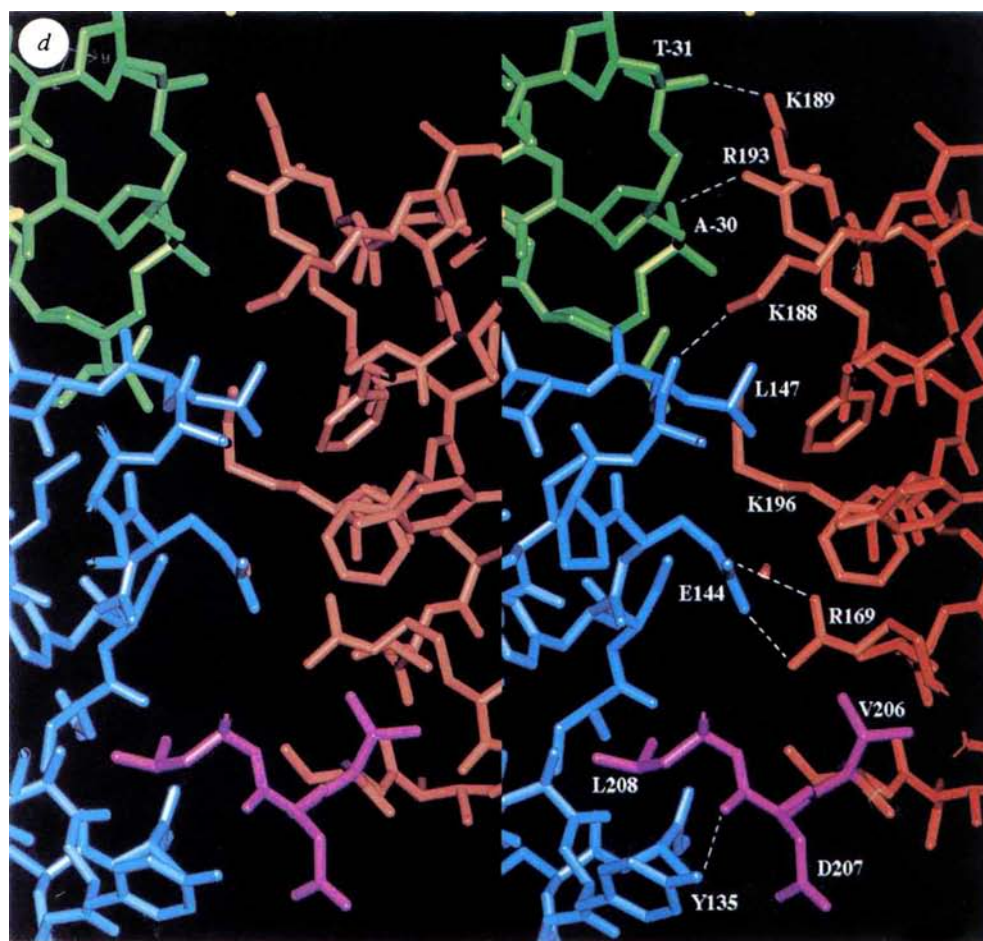


TBP2 is colour-coded white in this view, because the electron density is blue.

with cTFIIB are identical between plants, yeast, *Drosophila* and human with the single exception of Tyr 135, which is Phe in *Saccharomyces cerevisiae* and Leu in *Drosophila* and human (TBP sequence conservation is reviewed in ref. 5). Moreover, the 12 residues of human cTFIIB making contacts with TBP2 are also highly conserved (7/12 identical and 2/12 conservative substitutions between yeast, human and *Drosophila*).

The stability of the ternary complex comes from favourable van der Waals and electrostatic interactions between cTFIIB and TBP2 plus DNA (60% of TFIIB's buried solvent-accessible surface is either polar or charged, with the remainder hydropho-

bic). Figure 5 shows the basic surface of cTFIIB interacting with the acidic C-terminal stirrup of TBP and the negatively charged phosphodiester backbones of the DNA. It is therefore not surprising that TFIIB enhances the affinity of TBP for the core promoter³³. cTFIIB recognition of the TBP2-DNA complex is directional, exploiting both the asymmetry of the surface of TBP and the path of the phosphoribose backbone as it traverses across the underside of the molecular saddle. Some point mutants of TFIIB that interfere with ternary complex formation involve cTFIIB residues that interact with the DNA backbone in our structure (Figs 1 and 4). Although the asymmetric unit



of our cocrystals corresponds only to base pairs -34 to -19 of the AdMLP, DNA base stacking with neighbouring complexes permits us to visualize cTFIIB-DNA interactions consistent with contacts ranging from positions -37 to -14. Indeed, crosslinking of full-length TFIIB to the AdMLP has been

observed at position -19 (ref. 34), and TFIIB footprinting has been observed on either side of the TATA element¹⁸.

cTFIIB-TBP2 interactions can be divided into three classes. (1) TBP2's C-terminal stirrup constitutes the primary binding site for cTFIIB. Residues 143-147 participate in salt bridges,

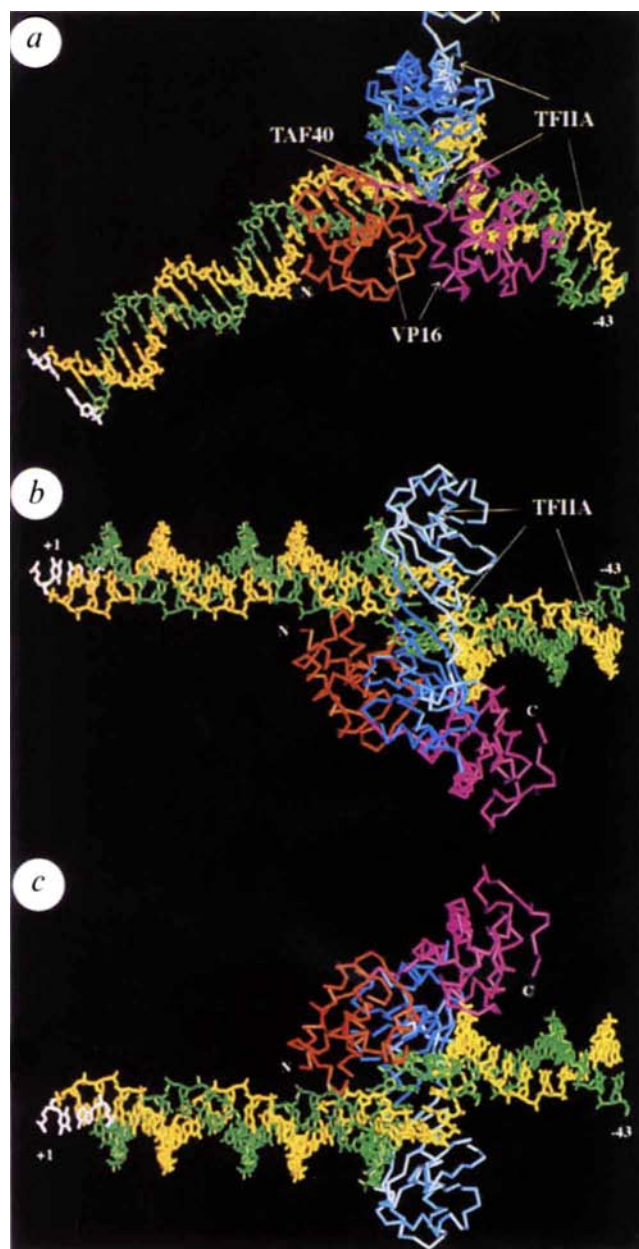


FIG. 3 TFIIB and TBP interacting with the AdMLP. The DNA is depicted as a stick figure, with hypothetical, linear B-form DNA extensions modelled at both the 5' and 3' ends of the X-ray structure oligonucleotide. The transcription start site is identified as the white base pair labelled +1. Sites of TFIIB crosslinking to the AdMLP are indicated, with the H2 surface of TBP implicated in TFIIB binding. TFIIF and TFIIB crosslink to the AdMLP in the vicinity of the N terminus of cTFIIB. Regions of core TFIIB implicated in interactions with VP16 and *Drosophila* TAF_{II}40 are also denoted. The region of cTFIIB that interacts with TAF_{II}40 overlaps with another VP16 binding site. *a*, View at 90° with respect to TBP's axis of approximate intramolecular symmetry, from above the saddle, showing the proximity of the first domain of cTFIIB and DNA 3' of the TATA element. *b*, View along TBP's axis of approximate intramolecular symmetry, from above the saddle, showing the proximity of the first domain of cTFIIB and DNA 3' of the TATA element. *c*, View as in Fig. 2c from below the molecular saddle, showing the proximity of the second domain of cTFIIB and DNA 5' of the TATA element.

hydrogen bonds and van der Waals (vdW) contacts (<4 Å) with at least 10 cTFIIB residues (Figs 2d and 5) (TFIIB residues are in italics), including Tyr 143→Leu 208 (vdW), Glu 144→Arg 169 (OE2-NH1=3.2 Å), Glu 144→Phe 195 (vdW), Pro 145→

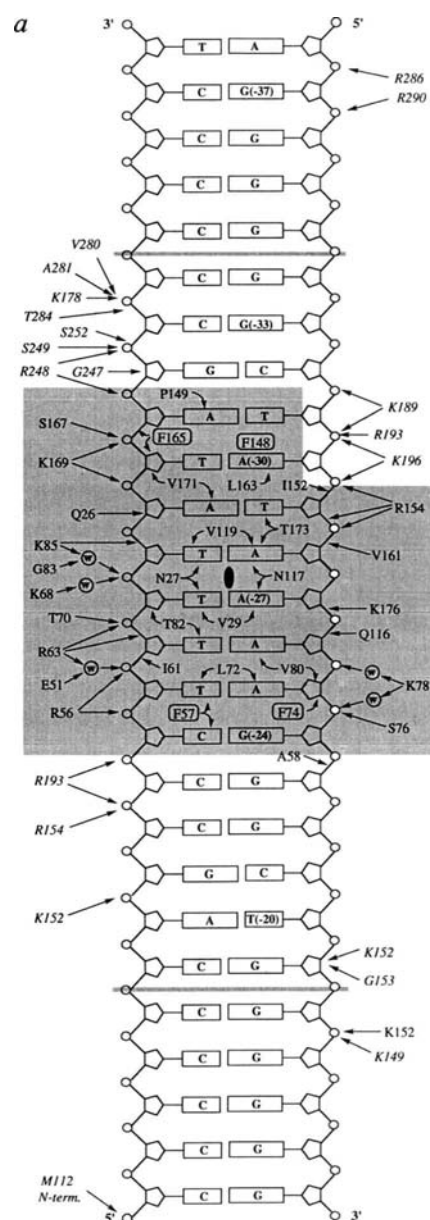


FIG. 4 Structural features of DNA binding and deformation. *a*, Schematic view of the cTFIIB-TBP2-DNA interaction. TBP2-DNA interactions are restricted to the shaded area and shown in roman type (water-mediated contacts have been inferred from ref. 11). TFIIB-DNA interactions are depicted in italics. The extent of the crystallization DNA is indicated with shaded bars. *b*, DNA structural parameters calculated with the program CURVES⁴⁹ using a local helix axis. DNA parameters for the 8-bp TATA box sequence are in bold. Corresponding values are given for the TBP2-DNA complex at 1.9 Å resolution¹¹, and for A- and B-form DNA. Minor groove width is defined as the shortest O4'-O4' distance minus 2.8 Å (the sum of the van der Waals radii of two oxygen atoms).

Ser 249 (vdW). Pro 145→Pro 250 (vdW), Glu 146→Tyr 165 (OE1-OH=3.5 Å), Glu 146→Thr 176 (vdW), Glu 146→Phe 177 (OE2-NH=3.2 Å), Glu 146→Lys 188 (O-NZ=2.6 Å), Leu 147→Phe 177 (vdW), and Leu 147→Gly 192 (vdW). (2) The solvent-accessible surface of α -helix H1' of TBP2 interacts with at least two residues from cTFIIB (Figs 2d and 5), including Glu 131→Leu 208 (vdW), Tyr 135→Asp 207 (OH-O=2.7 Å), Tyr 135→Asp 207 (OH-OD2=2.7 Å) and Tyr 135→Leu 208 (vdW). (3) Lys 197, located near the C terminus of TBP2, makes a salt bridge with Asp 243 (NZ-

Base pair	G C	G C	C G	T A	A T	T A	A T	A T	A T	A T	G C	G C	G C	C G	T A	G G	B	A
Helical twist (°)	35.5	30.3	36.8	19.9	19.9	26.3	11.2	20.3	19.5	23.9	36.8	34.2	36.4	34.0	39.1		36.0	30.7
TBP2-TATA		32.7	32.5	18.3	19.9	23.7	14.0	19.2	22.8	29.0	31.2	37.0	23.8	32.0				
Roll (°)	9.0	-1.4	6.9	38.9	20.4	11.1	23.8	21.0	26.5	30.8	6.6	0.4	-6.3	9.3	-3.3		0.9	11.4
TBP2-TATA		-5.4	3.1	40.2	21.4	11.4	25.2	22.0	16.0	44.8	4.4	3.0	-1.9	2.8				
Rise (Å)	3.52	3.21	3.61	4.95	3.62	3.34	3.68	4.01	3.87	5.31	3.20	3.69	3.66	2.92	3.04		3.38	3.44
TBP2-TATA		3.44	3.15	5.36	3.93	3.06	3.33	3.82	3.52	5.74	3.28	3.48	3.48	3.11				
Tilt (°)	-2.36	0.70	1.42	-2.70	4.04	2.34	-1.06	5.61	-5.84	-6.59	9.27	0.53	-1.77	13.84	-11.47		0.0	0.0
TBP2-TATA		0.68	4.57	0.19	2.26	4.20	-1.98	-0.97	2.01	-3.65	-1.85	-0.79	1.95	4.17				
Slide (Å)	-1.81	-1.29	-0.39	-1.13	-0.69	1.67	0.77	1.01	-0.18	0.22	-1.39	-1.83	-0.57	-1.22	2.35		0.1	-1.92
TBP2-TATA		-1.59	-0.54	-0.83	-0.80	1.66	1.16	1.04	0.59	0.18	-0.61	-0.90	-1.13	0.13				
P-P distance (Å)		5.98	5.99	5.74	6.74	5.57	5.73	5.92	6.37	5.56	7.13	6.92	6.21	7.13	5.74		7.0	5.9
TBP2-TATA	6.98	6.94	6.50	5.95	6.01	5.92	6.02	5.91	6.30	6.72	6.16	6.49	6.23	7.46				
		5.54	5.46	6.20	5.60	6.03	5.83	6.11	5.99	6.87	6.83	6.65	6.65	6.64				
		6.65	6.66	5.74	6.15	6.14	5.94	6.03	6.42	5.19	6.38	6.40	5.78					
Minor groove width (Å)	4.26	4.32	5.92	7.14	7.90	9.08	8.99	9.14	8.76	8.06	7.15	4.56	4.91	5.21	4.97		3.97	6.18
TBP2-TATA			6.47	7.35	7.90	9.10	8.83	9.07	8.44	7.64	7.28	5.90	6.17					
Buckle (°)	-6.4	-17.1	14.8	-13.0	-36.0	-18.5	1.2	14.7	27.1	34.5	17.2	26.9	3.6	-4.8	11.3	9.6	0.0	0.0
TBP2-TATA		-11.8	16.9	-12.6	-27.6	-24.1	-3.6	14.6	22.3	30.4	6.2	7.8	-0.9	2.8	7.7			

OD2=3.3 Å). The contact between Leu 147 and TFIIB was detected earlier via site-directed mutagenesis of this aliphatic side chain to a lysine, abolishing TFIIB binding to TBP (ref. 19). At 2.7 Å resolution, there are no features in the electron-density maps consistent with water molecules trapped in the cTFIIB/TBP2 interface.

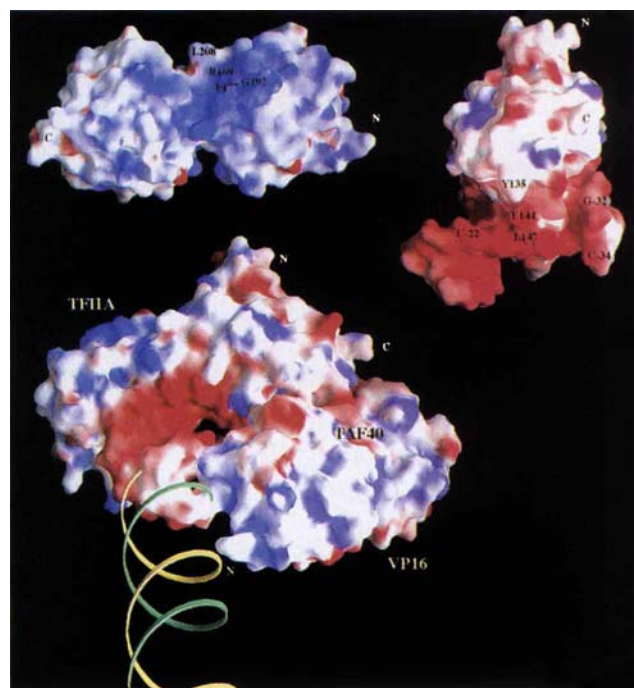
PIC assembly and transcriptional activation

The structure of the cTFIIIB-TBP2-DNA ternary complex has implications both for assembly and stabilization of the preinitiation complex. The marked TBP-induced bend in the core promoter is largely unaffected by TFIIIB binding. This unprecedented DNA deformation permits the general transcription initiation factors, pol II and transcriptional activators to have closer associations than would be feasible on linear DNA. Figures 3 and 5 show views of a model of cTFIIIB and TBP2 bound to the

AdMLP in which linear B-form DNA has been modelled at each end of the cocrystallization oligonucleotide. Regions of both TBP and the AdMLP have been implicated in binding TFI_{II}A (reviewed in refs 5, 34), which stabilizes the TBP-DNA complex^{33,35,36}. Crosslinking studies have localized a subunit of yeast TFI_{II}F to position -19 of the AdMLP, where TFI_{II}B also crosslinks³⁴ and a cTFI_{II}B-DNA contact is seen in our structure. Protease footprinting studies have identified proteolytically sensitive surface regions of TFI_{II}B, which map to the molecular surface and are protected by a pol II TAF (*Drosophila* TAF_{II}40 or its human homologue)³⁷.

Figure 5 illustrates a model of cTFIIB and TBP2 interacting with the AdMLP, viewed from +1 (the 'polymerase II view'). TFIIB plays a critical role in determining the transcription start site. Mutations in the N-terminal portion of TFIIB alter the start site³⁸. Given the crosslinking results described above, it

FIG. 5 Molecular surfaces of the TFIIB-TBP-DNA ternary complex. GRASP⁵⁰ molecular-surface representations of cTFIIB, TBP2 and the crystallization DNA, displaying the interaction surfaces. Solvent-accessible surfaces were calculated in GRASP with a water-probe radius of 1.4 Å, and coloured by electrostatic potential (red and blue surfaces represent electrostatic potentials in the range <-12 to $>+12k_B T$, where k_B is the Boltzmann constant and T is the temperature). Calculations were performed assuming an ionic strength of 0.1 M and dielectric constants of 80 and 2 for the solvent and protein/DNA, respectively. The upper diagrams show interaction surfaces of cTFIIB (left, viewed from TBP2's second stirrup) and the TBP2-DNA complex (right, viewed as in the upper panel of Fig. 3). The N and C termini of cTFIIB and TBP2 are labelled, as are key residues and nucleotides on the interaction surfaces. The lower diagram shows the solvent-accessible surface of the cTFIIB-TBP2-DNA ternary complex, viewed as in Fig. 2a (the 'polymerase II view'). The green and yellow ribbons correspond to the path of the phosphoribose backbone projecting from the 3' end of the cocrystallization DNA to the transcription start site in the AdMLP (+1), using the hypothetical DNA path from Fig. 3. The interaction sites on TBP of TFIIA, and *Drosophila* TAF_{II}40 and VP16 on TFIIB, are also labelled. The TAF_{II}40 interaction region has also been shown to interact with VP16.



is likely that the cTFIIB downstream surface contains a binding site or sites for pol II and/or TFIIF. The results of two studies support this prediction. First, human TFIIB will not substitute for yeast TFIIB, but human TFIIB plus human pol II will substitute for yeast TFIIB plus yeast pol II *in vitro*, although producing a different start site³⁹. Second, replacements of conserved residues in the large subunit of yeast pol II and mutations in yeast TFIIB have similar effects on start site selection⁴⁰.

TFIIB is also the target of a considerable number of regulatory factors, containing activation domains from various classes (reviewed in refs 2, 41). Among these, interactions between TFIIB and the acidic activation domains are reasonably well characterized. Protease footprinting studies have identified proteolytically sensitive surface regions of cTFIIB protected by the VP16 activation domain³⁷, and site-directed mutagenesis studies have implicated a TFIIB basic region (Arg 185–Lys 200) in interactions with VP16 (ref. 42). *Drosophila* TAF_{II}40 and its human homologue also interact with both TFIIB and VP16 during activator-dependent transcription^{43,44}.

The TFIIB–TBP–DNA ternary complex structure provides the first direct look at some of these transcription factor binding surfaces (Figs 3 and 5). Moreover, the structure provides a plausible explanation for the effects of an intriguing TBP mutation, mapping to the TBP2–TFIIB interface. A point mutation corresponding to Leu 147→Lys in yeast TBP both weakens TFIIB binding to the TBP–TATA element complex and selectively eliminates activator-dependent (but not basal) transcription. The mutant TBP cannot form a TFIIB–TBP–DNA (TB) complex, but can form a quaternary complex with TFIIA (TBA) supporting only basal transcription. The mutation maps to the interface between TBP2 and the first domain of cTFIIB, presum-

ably destabilizing van der Waals interactions between Leu 147 and *Phe* 177 and *Gly* 192 in cTFIIB. Assuming some inter-domain flexibility, this perturbation could disrupt interactions between domain one (red in Fig. 3) and the DNA, without affecting domain two (magenta in Fig. 3). The latter binds to the DNA backbone upstream of the centre of the TATA element, in the vicinity of known TFIIA crosslink sites³⁴, where it probably interacts with TFIIA in the TBA complex. Thus TFIIA could stabilize an energetically less favourable mutant TBP–TFIIB complex, permitting basal transcription. However, VP16 recognizes the two domains of TFIIB together³⁷, explaining a selective effect of the Leu 147→Lys mutation on activator-dependent transcription.

Conclusion

This study of the cTFIIB–TBP2–TATA element ternary complex provides the first established structure of TFIIB and the first structure of a protein recognizing the unprecedented TBP–DNA complex, explaining many of the biochemical and transcriptional properties of TFIIB and the TFIIB–TFIID (DB) and TB complexes. Our work also provides a starting point for further crystallographic, biochemical and genetic studies of the TFIIB–TBP–core-promoter assembly and its role in pol II-mediated transcription initiation. In particular, these data should aid directed, systematic analyses of DB and TB complex assembly, the mechanisms by which RNA polymerase II and TFIIF enter the growing PIC and establish the transcriptional start site, and recognition of the DB and TB complexes by transcriptional activators and coactivators. Finally, the similarity of cTFIIB and cyclin A suggests that TFIIB may modulate the activities of the kinase and cyclin subunits of TFIIF (ref. 45) or other cyclin-dependent kinases (reviewed in ref. 30). □

Received 28 July; accepted 21 August 1995.

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ACKNOWLEDGEMENTS. We thank S. Ramakoti for help with X-ray measurements; B. Chait and S. Cohen for the mass spectrometry at the National Resource for Mass Spectrometric Analysis of Biological Macromolecules; A. Ferré-D'Amaré, A. Hoffmann, H. Houbaviy, J. L. Kim, G. Patikoglou and P. Penev for many discussions; A. Gazes and the staff of Rockefeller University Computing Services for technical support; and J. Kuriyan and G. A. Petsko for many suggestions. D.B.N. is a Rockefeller University Graduate Fellow. This work was supported by the Howard Hughes Medical Institute (S.K.B.), and the NIH, Pew Trust and Human Frontiers Program (R.G.R.).

Discovery of a brown dwarf in the Pleiades star cluster

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BROWN dwarfs are cool star-like objects that have insufficient mass to maintain stable nuclear fusion in their interiors. Although brown dwarfs are not stars, they are expected to form in the same way, and their frequency of occurrence should reflect the trends seen in the birthrates of low-mass stars. But finding brown dwarfs has proved to be difficult, because of their low intrinsic luminosity. The nearby Pleiades star cluster is widely recognized as a likely host for detectable brown dwarfs because of its young age—the still-contracting brown dwarfs should radiate a large fraction of their gravitational energy at near-infrared wavelengths. Here we report the discovery of a brown dwarf near the centre of the Pleiades. The luminosity and temperature of this object are so low that its mass must be less than 0.08 solar masses, the accepted lower limit on the mass of a true star^{1–3}. The detection of only one brown dwarf within our survey area is consistent with a smooth extrapolation of the stellar mass function of the Pleiades⁴, suggesting that brown dwarfs, although probably quite numerous in the Galactic disk, are unlikely to comprise more than ~1% of its mass.

Only about half a dozen extremely cool dwarfs with spectral types M9 or later have been identified⁵. At present they are the best candidates for brown dwarfs. Their mass–luminosity and mass–spectral-type relationships^{6,7} show that these objects are indeed very close to the substellar limit, but uncertainties in their ages, metal contents and effective temperatures prevent an unambiguous assignment to stellar or substellar categories. If found in open clusters whose distances, ages and metallicities are well known, such late-spectral-type objects could provide a solution to the brown-dwarf dilemma. At the age of the Pleiades open cluster (70–120 Myr), brown dwarfs are still moderately luminous contracting objects that radiate a large fraction of their gravitational energy at red and near-infrared wavelengths. Previous photometric surveys of the Pleiades^{8,9} and follow-up spectroscopy have provided several brown-dwarf candidates earlier than spectral type M7. According to the lithium test^{10,11}, most of them are not truly brown dwarfs^{12,13}. However, evidence for the presence of lithium in one of the lowest-luminosity Pleiads (PPI 15) has recently been presented¹⁴, strongly suggesting that it is a transitional object located just on the borderline between stars and brown dwarfs. Hence brown dwarfs in the Pleiades cluster would be expected to be fainter and cooler than PPI 15.

We report here on the photometric and spectroscopic properties of a newly discovered M9 dwarf (one magnitude fainter in the I band than PPI 15) whose proper motion identifies it as a member of the Pleiades cluster. We have named it Teide 1 (hereafter referred to as Teide 1). This object, located at ~16 arcmin from the cluster centre, is significantly cooler and presumably less massive than any other previous cluster brown-dwarf candidates with measured proper motion and radial velocity. Teide 1 was discovered as a result of a continuing R-I-band CCD (charge-coupled device) photometric survey of the Pleiades (about 175 arcmin²) with the IAC-80 telescope at Teide Observatory on the island of Tenerife. The object coordinates are RA = 3 h 47 min 18 s, dec. = 24° 22' 31" (Eq. 2000, Epoch 1994.85). It was first found as a faint $I = 18.80 \pm 0.07$ mag red object ($R - I > 2.0$) and reobserved with the Nordic Optical 2.5-m Telescope at Observatorio del Roque de los Muchachos (ORM, La Palma) where CCD photometry yielded $(R - I)_c = 2.74 \pm 0.10$ mag. Our inspection of archive CCD images obtained¹⁵ in 1986 at the 2.5-m Isaac Newton Telescope (ORM), also revealed the presence of this previously unnoticed object,

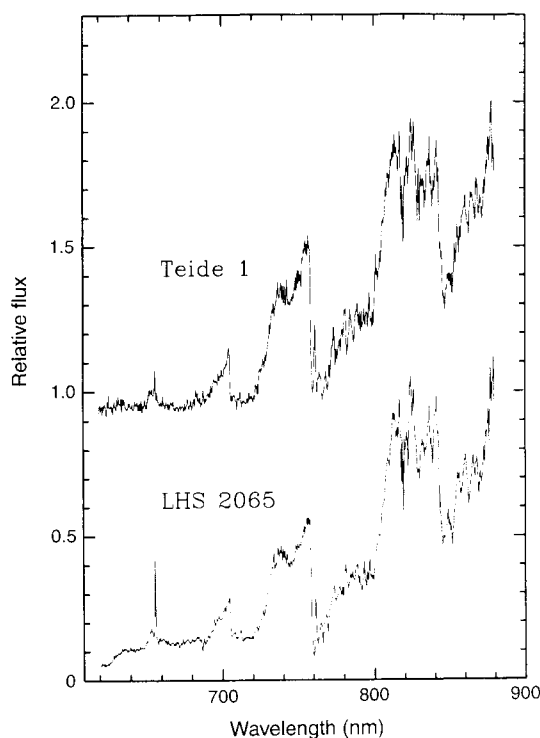


FIG. 1 The optical spectrum of Teide 1 (upper trace) and of the M9 dwarf LHS 2065 (lower trace). The flux scale of both spectra is normalized to unity at 825 nm. An offset has been added to the spectrum of Teide 1 for clarity.

allowing a determination of proper motion. From November 1986 to November 1994, the measured proper motion of Teide 1 was $\mu_{\alpha \cos \delta} = 0.13'' \pm 0.07''$ and $\mu_{\delta} = -0.28'' \pm 0.13''$, which is consistent within the error bars with that of the Pleiades cluster for the same time interval¹⁶ (that is $\mu_{\alpha \cos \delta} = 0.20'' \pm 0.05''$ and $\mu_{\delta} = -0.36'' \pm 0.05''$).

A spectrum of Teide 1 (shown in Fig. 1) with a resolution $\sim 6 \text{ \AA}$ was obtained on 29 December 1994 with the 4.2-m William Herschel Telescope (ORM) using the intermediate dispersion spectrograph ISIS with the 158R grating and a TEK 1,124 $\times$ 1,124 detector. We used a slit width of 1" oriented at the parallactic angle to minimize losses caused by differential atmospheric refraction. Several spectra with exposure times in the range 1,800–3,600 s were obtained, the total integration time being 16,600 s. Each individual frame was processed by standard techniques, and wavelength and flux calibrated within the IRAF environment. The r.m.s. error of the fourth-order polynomial fit to the emission lines of a CuNe lamp was 0.1 $\text{\AA}$. Also plotted in Fig. 1 is the spectrum of LHS 2065, a well-known M9 dwarf⁵, observed with the same instrumental configuration. Teide 1 is an extremely cool M dwarf with clear similarities to LHS 2065. Both have similar TiO and VO molecular bands as well as a sharply rising 'continuum' throughout the 630–880 nm spectral range. The strong lines of K1 at 766.5 and 769.9 nm and NaI at 818.3 and 819.5 nm in the spectrum clearly indicate that we are dealing with a high-gravity low-mass dwarf (the strength of these lines rules out the possibility that Teide 1 could be a background giant). H α is seen in emission, with an equivalent width of $6 \pm 1 \text{ \AA}$, a value common among very-low-mass Pleiades members^{17–20}. From the centroid of each of the lines of the NaI doublet in the individual spectra of Teide 1 we derived a heliocentric radial velocity of $v_r = 3 \pm 6 \text{ km s}^{-1}$, where the error corresponds to the mean of six spectra. An additional measurement of radial velocity was obtained from a higher-resolution spectrum of the NaI doublet acquired at the 4.2-m William Herschel Telescope on 1995 January 23 with the ISIS spectrograph and

the 600R grating (nominal dispersion $0.79 \text{ \AA}$ per pixel, effective resolution $1.6 \text{ \AA}$). Teide 1 was cross-correlated with the template²¹ GL 873, a dM4.5e star with $v_r = 0.47 \pm 0.24 \text{ km s}^{-1}$, yielding $v_r = 0.3 \pm 5.5 \text{ km s}^{-1}$ for our object. These two independent determinations are consistent with the mean radial velocity²² of 5.9 km s^{-1} for the Pleiades.

The strength of the VO band centred at 750 nm depends strongly on the effective temperature⁵ in stars with spectral types later than M7. We used the VO spectral index defined in ref. 5 (the ratio of the flux in the near pseudocontinuum at both sides of the VO feature to the actual flux observed in the minimum of the band) to derive the spectral type of Teide 1. We measured a value of 1.145, which corresponds to M9 (the range given in ref. 5 for this spectral type is 1.120–1.170). For LHS 2065 the same index gave 1.137, confirming its classification as M9. Using a spectroscopic database for very cool dwarfs kindly provided by J. D. Kirkpatrick, and a cross-correlation peak-technique, we determined a spectral type of $M8.5 \pm 0.5$ for Teide 1. This shows that both methods yield consistent classifications within the error bars. In addition, we found that the NaI doublet (very sensitive to spectral type in late M dwarfs) has an equivalent width of $6 \pm 1 \text{ \AA}$ in Teide 1, a value similar to those of field M9 dwarfs.

The measured proper motion and radial velocity, the H α emission, the dM9 spectral type and the RI photometry are all consistent with Teide 1's being a member of the Pleiades cluster. But the proper motions and radial velocities of Pleiades stars are similar to those of general field M dwarfs, as stated in ref. 18, so we considered the probability that Teide 1 might simply be a field M9 dwarf superimposed on the Pleiades (that is, lying at a distance of 60–100 pc, closer than the widely accepted cluster distance of 125 pc). The luminosity function at the end of the Main Sequence has been addressed recently as a result of a deep (completeness limit $R=19$), large-area (27.3 deg^2), CCD survey

for cool dwarfs²³. In this survey, only one extremely cool dwarf (spectral type M8.5) was found in a space volume of 412 pc^3 , suggesting that the local density of M8.5–M9 objects is $\sim 0.0024 \text{ pc}^{-3}$. Because our survey covered a volume about 80 times smaller, the probability of finding such late-spectral-type objects along the line of sight towards the cluster is $<1.5\%$. The discovery of our object can, however, be understood in terms of the much higher stellar density in the Pleiades cluster than in the field.

To estimate the mass of Teide 1 we had to determine its location on the Hertzsprung–Russell (H–R) diagram. The luminosity and effective temperature were derived from the I magnitude and the dM9 spectral type. Using the parameters of the Pleiades cluster (distance modulus of 5.53, extinction $A_I = 0.07 \text{ mag}$), and the I bolometric correction²⁴ suitable for LHS 2065, we obtained $\log L/L_\odot = -2.9 \pm 0.1$, where $L_\odot$ is the luminosity of the Sun, and the error comes from uncertainties in I magnitude, extinction, and distance. At present, there is no agreed temperature scale to place very-low-mass stars and brown-dwarf candidates in the H–R diagram. Therefore we adopted a mean temperature from several recent calibrations^{25–28} of $2,350 \pm 300 \text{ K}$. The error bar covers the total temperature range obtained with the different calibrations. In Fig. 2 we show the H–R diagram with a set of theoretical evolutionary tracks and isochrones¹ for stars close to the substellar limit, and for brown dwarfs. Superimposed are the dimmest Pleiades brown-dwarf candidates with measured proper motions and published spectral types^{12,13,29,30}. We have derived their luminosities and temperatures in the same way as for Teide 1. Our object is the coolest and faintest brown-dwarf candidate discovered so far in the Pleiades. From its location on the H–R diagram and after comparison with the set of theoretical tracks shown in Fig. 2 we obtain an upper limit to the mass of Teide 1 of 50 jovian masses (M_{Jup}) and a most likely mass in the range 20–30 M_{Jup} . If we use other sets of theoretical tracks^{2,3,31}, we always obtain masses $<70 M_{\text{Jup}}$. We conclude that Teide 1 must be a brown dwarf, or else the theory is seriously wrong.

Observational tests of the substellar nature of Teide 1 are valuable. Definitive proof would be the detection of lithium in the atmosphere. If its mass is indeed lower than $50 M_{\text{Jup}}$ as inferred from the theoretical H–R diagram, this object should not have destroyed lithium at all, in marked contrast with stars above the substellar mass limit, which have destroyed most of this element at the age of the Pleiades^{12,13}. Calculations of the formation of LiI lines in the atmospheres of very cool dwarfs^{10,11,32} indicate that fairly strong absorption features should be present at optical wavelengths. In particular, we predict on the basis of our estimated temperature for Teide 1, an equivalent width of $\sim 5 \text{ \AA}$ for the LiI 670.8-nm resonance doublet. The upper limit to the equivalent width inferred from our spectrum in Fig. 1 is too poor to constrain its lithium content. Detection of this line should be pursued with mid-resolution spectroscopy employing the new class of very-large-diameter telescopes.

We have presented compelling evidence for membership of an M9 dwarf in a young open cluster of known age. This allowed us to estimate an upper limit for the mass that is below the substellar borderline, and conclude that the body is a brown dwarf. It is encouraging that our survey to limiting magnitude $I=19$ ($R=21.5$), so far covering only $\sim 0.3\%$ of the total Pleiades cluster area, has provided one object of this nature. This was, in fact, what we had expected from a smooth extrapolation of the available mass function of the cluster⁴. This extrapolation predicts that 175 brown dwarfs in the mass range 80–40 M_{Jup} may be present in the cluster, forming one of the most populated mass intervals, even comparable to that of M-type dwarfs ($M \sim 0.5\text{--}0.08 M_\odot$) where 500 objects are expected. Although massive brown dwarfs could be quite numerous, their contribution to the total mass of the cluster is expected to be $\leq 10 M_\odot$, that is, $<1\%$. If the formation of stars and brown dwarfs in the

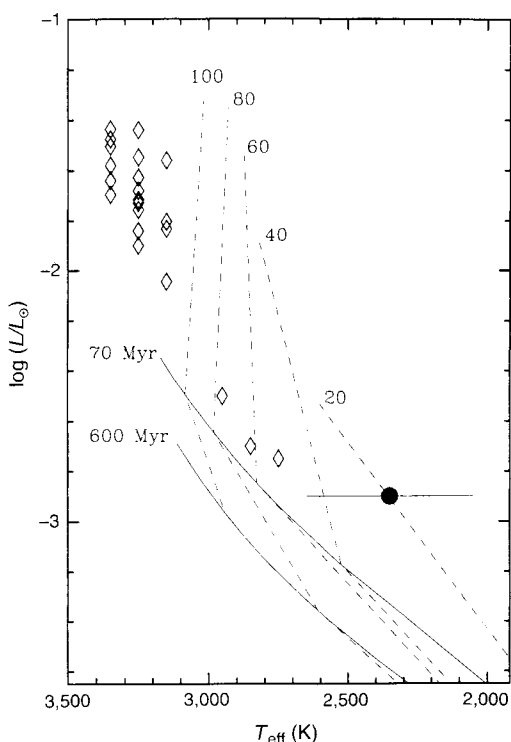


FIG. 2 The position of Teide 1 (filled circle) on the H–R diagram, with error bars corresponding to uncertainties in luminosity and temperature (see text). Other Pleiades proper-motion members with known spectral types are also located for comparison (open diamonds). Theoretical tracks¹ are plotted with broken lines and are labelled with approximate jovian masses. Two isochrones are plotted with solid lines.

Pleiades were representative of the Galactic disk, brown dwarfs would contribute no more than a few per cent to the disk mass, and it is therefore unlikely that they contribute significantly to the dark-matter problem. Other forms of baryonic dark matter should then exist if we are to account for the difference between dynamical and visible matter in galaxies. On the other hand, the number of brown dwarfs could be large enough for the basic properties of a new class of astronomical objects to be established in the near future. □

Received 22 May; accepted 14 August 1995.

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ACKNOWLEDGEMENTS. This paper is based on observations made with the IAC-80, NOT and WHT telescopes at the Observatorios de Instituto de Astrofísica de Canarias. We thank Nigel Hambly for his help with the proper-motion determination. Partial financial support was provided by the Spanish DGICYT.

Scaling of heating rates in solar coronal loops

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THE gas of the solar corona is at a temperature of several million degrees, orders of magnitude hotter than the underlying photosphere. The nature of the physical process that heats the solar corona (and the coronae of solar-type stars more generally) has been a long-standing puzzle. A number of plausible heating mechanisms have been proposed, but observations have so far been unable to discriminate between them¹. Here we show that coronal heating exhibits scaling properties that should provide a powerful diagnostic of the underlying mechanism. The coronal magnetic field organizes the coronal plasma into loop-like features, which form the basic structural elements of the corona². We demonstrate that the pressures and lengths of the coronal loops are statistically related, suggesting that the heating rate scales inversely with approximately the square of the loop length. Existing coronal heating theories make different predictions about what this scaling should be, and a model^{3,4} of energy dissipation by stressed coronal magnetic fields appears at present to be the most consistent with our observational result.

Coronal heating theories can be divided into two general categories: dissipation of waves and dissipation of stressed coronal magnetic fields. Within these two categories are a number of specific models, all of which are considered viable in the sense that they are theoretically sound and satisfy very basic observational constraints (for example, they produce enough energy to explain the observed radiation). The coronal heating problem is one of determining which of these contending models is correct. Unfortunately, many of the key differences between the models occur on spatial and/or temporal scales that are not observable from Earth, and so it is remarkably difficult to distinguish between them on the basis of remote sensing observations. This is a main reason why observational discriminators have been so scarce.

Soft X-ray and extreme ultraviolet images of the Sun reveal a clear tendency for the coronal emission to be concentrated in bright, arching loops, although there is also a diffuse component

that is presumably the cumulative effect of many fainter and less distinguishable loops. The loops are hot (generally 1–8 MK), have approximately uniform temperatures and pressures, and coincide with magnetic lines of force that are rooted to the solar surface at both ends. They lose energy by radiation to space and by thermal conduction down to the much cooler surface. Some loops are observed to evolve rapidly, but most have lifetimes that greatly exceed the timescale for cooling by radiation and conduction. It is reasonable to consider these loops to be heated quasi-steadily, meaning that the heating is truly constant or that the time interval between heating episodes is short compared with the cooling time. But another possibility that cannot be ruled out is that the loops are composed of many thin, unresolved strands that are each highly dynamic, but that together give the impression of a steady structure^{5,6}.

We have studied 47 apparently steady coronal loops observed by the Soft X-ray Telescope (SXT)⁷ flown on the Japanese Yohkoh satellite, an international project involving contributions from the USA and the UK. These loops were selected primarily because they could be relatively easily isolated from the non-uniform background emission by using the method of Klimchuk et al.⁸. The SXT has a complement of broadband analysis filters that give it valuable diagnostic capabilities. Using the filter ratio technique⁷, we compared the intensities observed through two different filters and determined the temperatures and emission measures (line-of-sight integrals of the square of the electron density) of the 47 loops.

We then determined the densities of the loops from the emission measures by assuming circular loop cross-sections, and thereby equating the line-of-sight thicknesses with the measured widths. We believe that this assumption is plausible because the solar photosphere is a turbulent fluid at observable scales (as evidenced by convective granules, for example), and it seems reasonable to suppose that similar motions also exist at subresolution scales corresponding to the photospheric feet of coronal loops. We can therefore expect most coronal loops to be twisted in the manner of a barber's pole. If a twisted loop had a non-circular cross-section, its width would appear to vary along its length. But in reality loops have remarkably uniform widths⁸, implying that the cross-sections are approximately circular.

From the measured densities and temperatures we determined the pressures of the loops from the ideal-gas law. These pressures are plotted in Fig. 1 against the measured loop half-lengths. We corrected for projection effects in determining the loop lengths by assuming that the loops are semicircular and taking the footpoints to be the locations where the intensity falls to 1/e of its maximum value. This procedure underestimates the true lengths

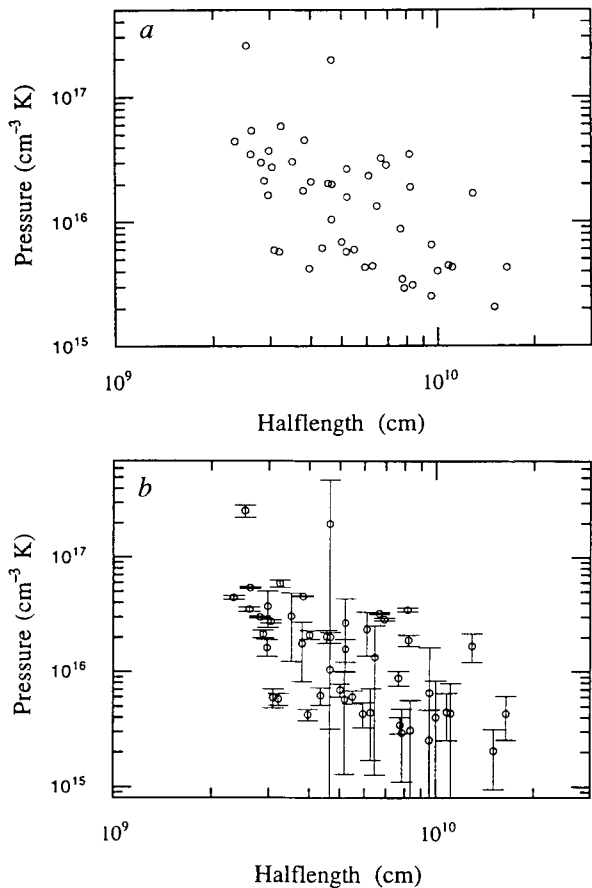


FIG. 1. Plots of pressure against halflength for the 47 loops. *a*, Measurements without error bars; *b*, measurements with error bars indicating the pressure uncertainties due to photon-counting statistics and data compression.

of some loops and overestimates the true lengths of others, but it should have little effect on our statistical results.

It is apparent from Fig. 1 that the pressures, which cover 2 orders of magnitude, are inversely correlated with the lengths. To quantify this, we performed a nonparametric (rank-ordered) statistical analysis using the weighted *t*-statistic^{9,10}. (Simulations show that SXT filter ratio measurements are not distributed normally, and therefore least-squares techniques, including the commonly used Pearson correlation coefficient, might produce spurious results.) Our analysis indicates that there is only a 4.07×10^{-3} probability that the data of Fig. 1 are uncorrelated, and we therefore conclude that pressure and length are correlated to a high degree of certainty. Furthermore, if

$$P \propto L^\beta \quad (1)$$

then β has a most probable value of -0.96 and a 90% confidence interval of $-1.82 \leq \beta \leq -0.22$, meaning that there is a 90% probability that β falls somewhere within this range. The possible effects of random and systematic errors in the pressure and length measurements have been taken into account in arriving at this result. Complete details of the analysis can be found in ref. 11.

Because a plasma's pressure is proportional to its thermal energy density, equation (1) is an important indicator of how the heating of a loop depends upon its length. This is true whether the loop is evolving slowly or rapidly, but for quasi-steadily evolving loops, we are able to make specific and powerful inferences. For such loops, the three primary sources and sinks of energy—heating, radiation and thermal conduction—are all of the same order¹², and it can be demonstrated that the

volumetric heating rate averaged along the loop axis, $\langle Q \rangle$, is related to the loop pressure and length according to

$$\langle Q \rangle \propto P^{1/4/(11-2b)} L^{(4b-8)/(11-2b)} \quad (2)$$

where b is the power-law index of the dependence of the radiation loss rate on temperature: $\mathcal{L}_r \propto T^b$. This result has been derived previously for the particular choice $b = -\frac{1}{2}$ (refs 13, 14), but recent calculations (J. Raymond, personal communication) indicate that $b \approx -\frac{3}{2}$ for $6.2 \leq \log T < 6.55$ and $b \approx \frac{1}{3}$ for $6.55 \leq \log T \leq 6.9$ is more appropriate. Combining equations (1) and (2), we obtain

$$\langle Q \rangle \propto L^\alpha \quad (3)$$

where

$$\alpha = \frac{2(7\beta + 2b - 4)}{11 - 2b} \quad (4)$$

Thus, by determining the value of β from observations, we are able to infer the value of the fundamental parameter α . Using the results above and taking $b = \frac{1}{3}$ (since $\log T \geq 6.55$ for most of our loops), we find that α has a most probable value of -1.95 and a 90% confidence interval of $-3.11 \leq \alpha \leq -0.95$. Note that α is only weakly dependent upon b , so that $b = -\frac{3}{2}$ produces a similar result.

Let us now compare this observed value of α with the values predicted by three specific models of coronal heating. Two of the models involve the dissipation of stressed coronal magnetic fields, and one involves the dissipation of waves generated at the coronal base. The first model was proposed originally by Parker^{3,4}. He pictures random motions of magnetic footpoints in the photosphere as causing a winding and wrapping of coronal magnetic flux tubes, the result resembling a bowl of tangled spaghetti. This process pumps energy into the field and induces coronal electric currents, especially at the boundaries between the flux tubes. As the angle between adjacent flux tubes increases, the currents grow, and there may exist a critical angle that cannot be exceeded owing to the onset of dissipative effects (such as tearing-mode instability, fast magnetic reconnection, or current-driven instabilities¹⁵). Footpoint motions continue despite the dissipation, and a quasi-steady state may be achieved in which the dissipation and energy input are balanced over a time interval that is short compared with a cooling time. If we associate coronal loops with the flux tubes in this picture, and if we assume that the footpoint motions are statistically independent of loop length, then we expect $\alpha = 2\delta - 1$, where δ is the power-law index of the dependence of the coronal magnetic field strength on loop length:

$$B \propto L^\delta \quad (5)$$

The observed value $\alpha \approx -2$ is reproduced if $\delta \approx -0.5$.

We next consider a variation on this model that involves a steady twisting of individual coronal loops by long-lived vortical motions at their footpoints¹⁶⁻¹⁸. We imagine that a critical twist exists, analogous to the critical angle above, and that some of the magnetic free energy stored in the stressed field becomes liberated when the critical state is reached. Certain macroscopic instabilities, such as the ideal and resistive kink instabilities¹⁹, are known to be triggered at a critical level of twist. These instabilities might lead to a concentration of some of the large-scale electric currents that are present^{20,21}, and these concentrated currents would then dissipate to heat the plasma. As long as the loop hovers near the critical state, the time-averaged heating rate will be such that $\alpha = 1.5\delta - 2$, where it is assumed that photospheric velocities, photospheric vertical field strengths, and coronal diameters of loops are statistically independent of loop length. If coronal diameters scale as $d \propto L^{0.2}$, as our data indicate, then $\alpha = 1.5\delta - 1.8$. In either case, the observed value of α is reproduced to within the uncertainties if $\delta \approx 0$.

Lastly, we consider one of a different class of models involving the dissipation of waves generated outside the loop, presumably

by turbulent motions in the photosphere and/or chromosphere. Although the generic concept of wave heating in the solar atmosphere is quite old, recent attention has focused on the resonance absorption of Alfvén waves (transverse magnetic waves) in coronal loops^{22–24}. In this model, the waves with frequencies close to the global oscillation frequency of a loop are able to propagate into the loop and dissipate in a layer where certain local conditions are met. On the basis of recent numerical simulations²⁵, and with the assumption that the frequency spectrum of input wave power has the form $W(\omega) \propto \omega^m$, we find that the observed scaling is reproduced if $m(1-2\delta)-6\delta=7$. Although the spectrum of waves entering coronal loops is not known, it is reasonable to consider the value $m=-1$ appropriate to Kolmogorov turbulence⁴. In that case, we require $\delta \approx -2$ to reproduce the observed value of α (for a flat spectrum, $m=0$, we require $\delta \approx -7/6$).

We see that these three particular models of coronal heating predict three different values for magnetic index δ : -0.5 , 0 and -2 . It is not surprising that all three models depend on δ , because every coronal heating theory involves the magnetic field in some role, either active or passive. Fortunately, the dependence on the field is different in each case. Although the actual value of δ has not yet been determined observationally, we know that it must be negative, because magnetic fields weaken with distance from their source, and we know that its magnitude must be <3 , the limiting point dipole value that pertains only at great distances. Golub *et al.*¹⁸ have examined active regions of different sizes and found that the average pressure in an active region is related to its average field strength according to $\langle P \rangle \propto \langle B \rangle^{1.6}$. Combining this result with our result for the pressure/length scaling of individual loops suggests that $\delta \approx -0.7$. This is a point in favour of the Parker^{3,4} heating model, although it seems premature to rule out other models on the basis of this evidence alone.

The ideal way to determine δ observationally would be to make direct measurements of the magnetic field strength within loops. But measurements based on the Zeeman effect are very difficult because the magnetic splitting of ultraviolet and optical emission lines is usually much smaller than the thermal broadening at coronal temperatures. The possibility of using infrared lines, which have much larger splitting, is only now being explored²⁶. Coronal field strengths have been inferred from microwave observations of gyroresonance emission, but the uncertainties in these measurements are quite large. A new-generation Solar Radio Telescope, currently under consideration, might reduce these uncertainties considerably²⁷.

As a consequence of these difficulties, most previous attempts to determine the strength of coronal magnetic fields have relied on upward extrapolations of the more accurately measured photospheric fields. Early extrapolation models were of questionable value, but recent advances have greatly enhanced the usefulness and reliability of this technique. Traditional vector magnetographs observe the polarization of photospheric absorption lines in a single spectral bandpass, and inferring the magnetic field requires assumptions about the properties of the line forming region of the solar atmosphere. New instrumentation, such as the Advanced Stokes Polarimeter²⁸, fully resolve the line profiles and therefore are much less dependent on these assumptions. They are able to measure the field with a several-fold increase in accuracy²⁹. New methods for extrapolating the measured fields into the corona have also been developed recently^{30,31}. These methods are much more robust than previous ones, which tended to make unrealistic assumptions about coronal currents, to produce spurious results at high altitudes, or to fail altogether on fields with appreciable yet realistic stress.

A determination of δ has never before been attempted, most probably because its importance has only now become apparent. We believe that a reliable value could be determined from state-of-the-art extrapolation models based on the best available data, and we strongly advocate that such a study be undertaken

immediately. Armed with the scaling results presented here, we would then be in a position to draw definitive conclusions about the viability of competing models of coronal heating. This would represent a major advance in our understanding of solar and stellar coronae. □

Received 22 February; accepted 14 August 1995.

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ACKNOWLEDGEMENTS. We thank S. Antiochos, P. Cargill, J. Davila, T. Lee, L. Ofman, R. Rosner, G. Roumeliotis and P. Sturrock for discussions. Much of this work was performed while the authors held positions at the Center for Space Science and Astrophysics at Stanford University. This work was supported by NASA and the Air Force Office of Scientific Research. L.J.P. was supported by the ARCS foundation.

Possible coexistence of *s*- and *d*-wave condensates in copper oxide superconductors

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SINCE the discovery of superconductivity in the layered copper oxide materials¹, a number of microscopic models have been proposed. To know which of these should be considered further, it is important to determine empirically the symmetry of the superconducting order parameter (the wavefunction of the superconducting condensate). For some time there has been conflicting experimental evidence as to whether the superconducting condensate has *s*- or *d*-wave symmetry²; these terms, strictly correct only in tetragonal symmetry, are commonly used to denote whether the superconducting gap is finite in all directions at zero temperature or contains nodes. Tunnelling data along the *c* axis of these quasi-tetragonal copper oxides, perpendicular to the CuO₂ planes, clearly show an *s*-wave character^{3,4}. Conversely, tunnelling along the *a* or *b* axis of YBa₂Cu₃O_{7- δ} has indicated a *d*-wave character of the wavefunction^{5–7}, with one exception⁸. Here I propose that these and other apparently conflicting results can be explained in a consistent way if there exist in the copper oxide superconductors

two condensates, with different symmetry but the same transition temperature—in other words, if there are two kinds of superconducting gap.

The above-mentioned possibility is less far-fetched than one might think at first; after all, two gaps with the same transition temperature (T_c) have been observed in *n*-type SrTiO₃ (ref. 9), the first oxide in which superconductivity was detected¹⁰. Because the copper oxides have a small coherence volume, one might expect a similar behaviour. Indeed, in YBCO the presence of two gaps has been inferred theoretically from a number of experiments¹¹. The main gap would result from the CuO₂ planes, and an induced smaller one would be due to the CuO chains. In contrast to the present assumption, however, both would be nodeless. With two condensates of *s* and *d* type, the two kinds of experiment, namely tunnelling along the *c* axis or probing the condensate in the CuO₂ planes, somehow have to discriminate between the two different superconducting wavefunctions. We know that the measured coherence lengths in these cuprates are highly anisotropic: 1.5–3 Å along the *c* axis and 12–13 Å parallel to the CuO₂ planes¹². This difference entails an important consequence for tunnelling experiments, as pointed out in ref. 13.

By tunnelling along the *c*-axis, for a copper oxide with exact $d_{x^2-y^2}$ -wave superconductivity, no critical currents can be observed. The tunnelling currents cancel, owing to the positive ($+x^2$) and negative ($-y^2$) signs of the phases for this condensate perpendicular to the *c* direction (here the *z* direction is taken parallel to *c*). But this is not the case for the *s*-wave part of the condensate. A tunnelling current has to be present as observed³, but its magnitude is considerably reduced because it is proportional to the product of the superconducting gaps $\Delta(0) \cdot \Delta'(0)$ on both sides of the junction near T_c in superconductor (S)–insulator (I)–superconductor (S') (SIS') arrangement¹³. Now the gap of the high- T_c superconductor at its SI boundary, $\Delta(0)$, is depressed by $\Delta(0)/\Delta \approx b/\xi(T)$, where Δ is the bulk pair potential and *b* is the so-called extrapolation length (see Fig. 1 of ref. 13). In the case of a short coherence length, the approximate relation $b \approx \xi_0^2/a$ (ref. 13) holds, with ξ_0 being the zero-temperature coherence length and *a* the lattice constant. For $\xi_0 \ll a$ it follows that $b \ll \xi_0$, resulting in a pair potential at the interface of $\Delta(0)/\Delta \ll 1$.

In the first tunnelling experiment³ mentioned, the superconductor was covered by an unspecified insulator 'I' over which a 10-Å-thick silver barrier layer was evaporated, followed by a layer of lead (S') (ref. 3). This junction is accordingly denoted by SINS'. As discussed above, the gap is depressed at the SI interface to ξ_0/a at low temperature. With $\xi_0 = 2.5$ Å and *a* the lattice constant along the tetragonal axis of 12 Å, this results in a depression of a factor of five. We do not know the thickness of the oxide barrier, but an order of magnitude larger than ξ_0 is a good estimate. On top of the oxide comes the silver layer. Both layers depress the relevant gap further. In the present case both high- T_c gaps are depressed by more than an order of magnitude and are not visible in Fig. 1 of ref. 3. The critical current of the only allowed *s*-wave part at low temperature is then reduced by the square root of the gap depression (Ambegaokar-Baratoff formula—see equation 1 of ref. 14, that is, by about an order of magnitude³. In fact, arguing backwards from the measured currents, the *s*-wave gap must be depressed by about two orders of magnitude.

When letting a scanning tunnelling microscope tip punch into the copper oxide semiconducting surface or in the bulk⁴, there are two possibilities: the metallic tip is either in contact with a superconducting CuO₂ plane or a semiconducting layer, or perhaps with a poorly conducting CuO chain. For this kind of junction Deutscher and Simon¹⁴ have derived the following formula for the ratio of the extrapolation length *b* to the high- T_c superconductor coherence length ξ_S :

$$\frac{b}{\xi_S} = \frac{\xi_S}{d_N} \frac{N_S(0)}{N_N(0)} \quad (1)$$

Here d_N is the thickness of the normal conductor, and $N_S(0)$

and $N_N(0)$ are the density of states at the Fermi level of the high- T_c and normal conductors, respectively. Using $d_N = 2.5$ Å and $N_S(0)/N_N(0) \leq 10^{-2}$, one obtains with $\xi_S = 2.5$ Å, $b/\xi_S = 10^{-2}$. Consequently the high- T_c gap is depressed by more than two orders of magnitude. I have thereby assumed that, as a result of punching, the thickness d_N is of the order of the coherence length ξ_S . This will also be the case if a contact is made with a semiconducting or poorly conducting CuO chain of the oxide, but in this case $N_S(0)/N_N(0)$ can become considerably larger than unity. Therefore it follows from equation (1) that the extrapolation length can be larger than the coherence length, and almost the full gap becomes observable in the dI/dV data, as reported⁴.

We now turn our attention to the experiments with tunnelling perpendicular to the *c* axis—that is, along the *a* or *b* directions of orthorhombic YBCO. The measured coherence length along these two directions is about five times as large as that along the *c* axis¹². Because I have assumed that two gaps exist, two coherence lengths have to be present as well: one for each condensate. For a not too anisotropic *s*-symmetry component of the total superconducting wavefunction and the very short coherence length ξ_S along the *c* axis, the substantially longer ones along the *a* or *b* axis have to be due to the *d*-wave part. Consequently, tunnelling along the latter two directions should be dominated by the *d*-wave component, and using junctions along the *a* and *b* axes in a SQUID arrangement should detect the *d*-wave component, as is reported^{5,6}. The jitter in the data, especially in those of ref. 5, may result from small *s*-wave components (of the order of 10%) due to either one of the two junctions. I estimate this from the depression characteristics as outlined above, assuming the two components of the condensate to be comparable. This will be discussed further below.

With the two proposed superconducting order parameters, it also seems possible to explain the in-plane tunnelling between two superconducting Y₂Ba₂Cu₃O_{7- δ} films⁸. One of the films was patterned as a hexagon and its crystallographic axes were rotated in the plane of the film by 45° with respect to those of the other film. In this orientation the critical current at any particular junction is at a minimum, two orders of magnitude lower than it would be if the orthorhombic axes of the two films were aligned (with a rotation angle of 0°)¹⁵. It seems plausible that this very strong reduction in critical current pertains to the tunnelling of the *d*-wave condensate. The *s*-wave part will also be reduced, but less so. The experiment indeed proves this assumption, because only an *s*-wave tunnelling current is observed for all six junctions of the hexagon. In other words, the special orientation of the two YBCO films in the experiment of ref. 8 favours the *s*-wave component nearly completely over the *d*-wave component. Its measured critical current density was only 2×10^3 A cm⁻².

When rotating the crystal axes on the two sides of a junction by an angle smaller than 45°, the tunnelling current increases substantially¹⁵. This may reflect the dominance of the *d*-wave component in this geometry: in a tri-crystal ring experiment, Kirtley *et al.*^{7,16} observed half-integer flux quantization, characteristic of *d*-wave symmetry. The rotation angle of their junctions was nearly 30°, and their critical current density was 1.5×10^5 A cm⁻², a factor of 75 larger than in the 45° experiment⁸.

So far I have discussed tunnelling experiments, because these are the most relevant for determining the condensate ground state. I have selected the most recent experiments, and those that I feel to be most reliable; others are referenced in the cited papers. There are of course other methods that yield information on the superconducting symmetry, but they are more indirect. One example is the study of NMR spin relaxation times T_2 , which are anisotropic below T_c . From these data, Imai *et al.*¹⁷ very early deduced the possible existence of *d*-wave symmetry. The measured anisotropy was intermediate relative to those computed theoretically for *s* or *d*-wave pairing by Bulut and

Scalapino¹⁸. From this one can conclude that the two components have to be comparable, as mentioned earlier. How large the ratio of the two components is has to await a more detailed analysis.

On the basis of the above discussion, it is probable that NMR T_2 relaxation data integrate over the two components present in the condensate. This also appears to be the case for the recent angle-resolved photoemission experiments of Ma *et al.*¹⁹ in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_{8+x}$, in which the data are said to be compatible with two-component order parameter models (references to early and recent theoretical models are cited in ref. 15). In contrast, specific-heat experiments are sensitive to the presence of nodes²⁰ in the condensate, and therefore probe only the d component. The early longitudinal NMR relaxation data, $1/T_1T$, in YBCO-123 could be accounted for by d -symmetry^{21,22}. But the T_1 asymmetry between $0.5 < T/T_c < 1$ is difficult to analyse in YBCO-123 (ref. 22) and in YBCO-124 (ref. 23), owing to the minimum observed in both crystals. Radio-frequency measurements on film surfaces have been interpreted more recently as proof for the existence² or absence²⁴ of nodes in the condensate.

Thus, the best recent tunnelling data in the superconducting copper oxides can be quantitatively accounted for by assuming that two order parameters of s - and d -wave symmetry are present. This assumption also involves two coherence lengths that differ by nearly an order of magnitude. Because of this difference, specific tunnelling experiments differentiate clearly between the two order parameters. As outlined above, other types of experiment either integrate or differentiate between the two components.

A certain mixing of the two components of the superconducting ground state will be present, even at low temperature,

because the cuprates do not have a tetragonal structure. This mixing will be enhanced on increasing the temperature, as the two coherence volumes in question become larger. This may account for the existence of only one T_c for the two condensates. □

Received 16 June; accepted 15 August 1995.

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ACKNOWLEDGEMENTS. I thank Hugo Keller for a critical reading and corrections of this note, which was in part written while a Regent Lecturer at UCLA and the Lawrence Berkeley Laboratory, as well as a guest of Tel Aviv University and its Sackler Institute. These stays were promoted by Vladimir Kresin and Amnon Aharony, respectively. A thorough discussion with Guy Deutscher regarding ref. 14 is especially acknowledged.

Fully collapsed carbon nanotubes

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It has been suggested¹ that the tensile strength of carbon nanotubes² might exceed that of other known fibres because of the inherent strength of the carbon–carbon bond. Calculations of the elastic properties of nanotubes confirm that they are extremely rigid in the axial direction and are most likely to distort perpendicular to the axis^{3,4}. Carbon nanotubes with localized kinks and bends^{5,6}, as well as minor radial deformations^{7,8}, have been observed. Here we report the existence of multi-shelled carbon nanotubes whose overall geometry differs radically from that of a straight, hollow cylinder. Our observations reveal nanotubes that have suffered complete collapse along their length. Theoretical modelling demonstrates that, for a given range of tube parameters, a completely collapsed nanotube is favoured energetically over the more familiar ‘inflated’ form with a circular cross-section.

Carbon nanotube samples were prepared by a carbon-arc discharge method⁹. Our synthesis chamber employed a water-cooled 0.625-inch graphite anode, a water-cooled copper cathode, 650 Torr of environmental helium pressure, a 120 A d.c. electrode current, and an electrode gap of a few millimetres. The carbon growth on the negative electrode was removed from the synthesis chamber and baked in flowing oxygen at 650 °C for 30 minutes to remove excess carbon particles^{10–12}. Pieces of the baked sample were then manipulated by a glass slide and

mounted on holey carbon grids for analysis by transmission electron microscope (TEM). Samples were analysed on a JEM JEOL 200CX TEM with 200 keV accelerating voltage.

The majority of the structures imaged by TEM were consistent with previous observations: multi-walled cylindrical nanotubes with lengths varying from tens of nanometres to several micrometres, and multi-shelled ‘onions’. But we also observed long, ribbon-like structures distinctly different from conventional nanotubes. Figure 1 shows an example of such a structure, iden-

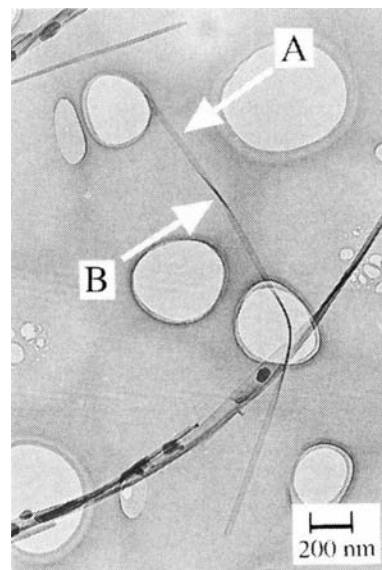


FIG. 1 TEM image of a collapsed carbon nanotube. Arrows point to flat (A) and twisted (B) regions of the collapsed tube.

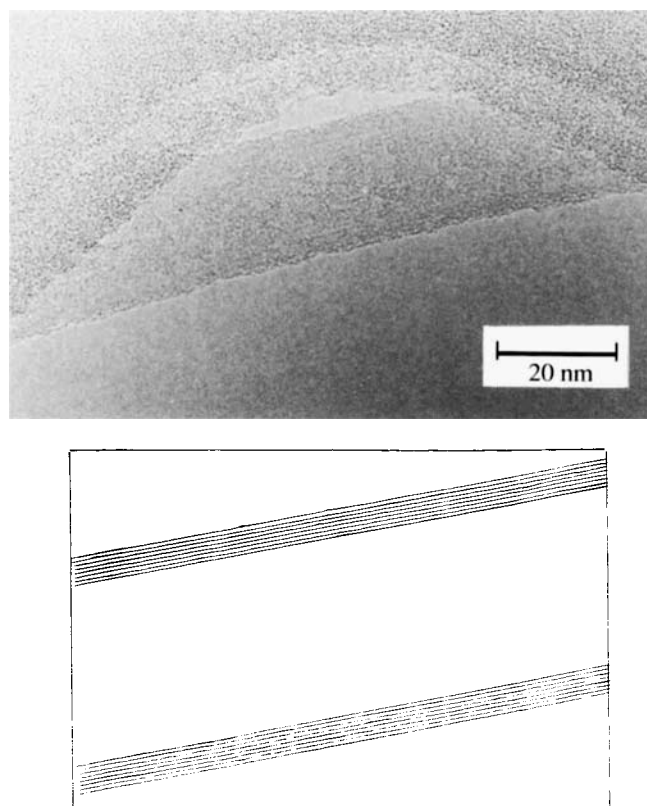


FIG. 2 High-resolution TEM image of flat region of a collapsed nanotube. The line drawing (not to scale) helps to clarify the image. Notice the equal number of lattice fringes ($n=8$) on each side of gap.

tified by arrows. On initial examination, this structure looks like a flat ribbon of width 22 nm and total length 2.4 μm , with two distinct twists along its length. A region of the structure apparently lying 'flat' against the grid substrate is marked A in the figure. One of the twists occurs over a hole in the support grid; the other twist is marked B in the figure. Other examples of such ribbon-like structures have been identified in our TEM images. We propose that these ribbon-like structures are in fact fully collapsed, flattened carbon nanotubes. We have performed additional high-resolution TEM imaging of the flat and twisted portions of several ribbon structures, and a sample rotation study, all of which support the identification of flattened tubes.

The TEM signature of a (conventional) hollow cylindrical multi-walled carbon nanotube is the observation of two parallel sets of equal-numbered lattice fringes, separated by a gap. The fringes represent the tube walls and the gap represents the hollow interior of the tube. A collapsed or flattened nanotube has a related but distinct signature. If the flat (wide) part of the tube is in the imaging plane, two parallel sets of equal-numbered lattice fringes, separated by a gap, should again be observed. If the flat part of the collapsed tube is perpendicular to the imaging plane, the number of distinct fringes should remain unchanged but the gap should be absent.

Figures 2 and 3 show this to be the case for the ribbon-like structures. Figure 2 shows a high-magnification image of the flat portion (analogous to region A in Fig. 1) of a ribbon structure different from that shown in Fig. 1. Two sets of 8 parallel lattice fringes are observed, separated by a gap of 16.2 nm. We label the inner gap width of a flattened tube as W_{in} . The full outer width of the flattened tube is W_{out} . The spacing between tube walls is calculated as about $(W_{\text{out}} - W_{\text{in}})/(2 \times 8) \approx 3.3$ Å, consistent with the interplanar spacing of graphite (and the wall separation of conventional multi-walled nanotubes). Figure 3 shows, for the same flattened tube as in Fig. 2, a high-resolution image of a twist region. In the centre of the twist region, the flat part of the tube is perpendicular to the image plane, and here a total of 16 lattice fringes, with no gap, are observed. The greater contrast of the lattice fringes in the twist region than in the flat region is consistent with the analysis of the structure as a flat tube. The thickness t of the tube in this region is 5.3 nm ($\sim 16 \times 3.3$ Å): t represents the 'height' of the flattened tube.

The 'twisted' regions of flattened tubes provide a convenient view of different tube orientations. However, to rule out the possibility that the ribbon structures we observe are only flattened in the twisted regions (and perhaps more like conventional, cylindrical tubes elsewhere), we performed a careful sample rotation study. The entire specimen grid was rotated *in situ* inside the TEM through angles in excess of 30° . Both ribbon structures and a 'control' conventional nanotube (located nearby on the same grid) were imaged. The control showed no change when tilted along an axis parallel to the tube axis, whereas the ribbon structure displayed a change in apparent width of the 'flat' region, consistent with a fully collapsed tube.

We have characterized a number of collapsed nanotubes in terms of number of walls n and characteristic dimensions. Table 1 summarizes the results. All collapsed tubes that we have clearly identified have n between 6 and 9, and flattened outer widths W_{out} of ~ 20 nm. Table 1 also lists calculated dimensions for the inner diameter D_{in} and outer diameter D_{out} that the tube would have if it were 'inflated' and thus assumed a circular cross-section.

It is interesting to speculate how fully collapsed nanotubes come into being. It is possible that they form when a conventional hollow cylindrical nanotube is locally deformed (for example, kinked or twisted) by external mechanical forces. On kinking, the inner tube wall collapses locally and starts a zipper effect, which then flattens the tube down its entire length. The van der Waals attraction between opposing and flattened inner walls may act as an adhesive keeping the tube stable in this new position. Every fully collapsed tube that we observe contains at least one twist. This may be evidence that the twists are the origin of tube collapse. Alternatively, it may be that collapsed nanotubes are prone to twisting, much as any flat ribbon is more prone to twisting than a hollow cylindrical tube of the same material. The similarity of tube parameters for collapsed nanotubes in Table 1 suggests that a particular class of tubes is susceptible to collapse and that tubes with a greater number of walls or a smaller inner diameter than those in Table 1 have greater structural integrity.

The stability of a flat, fully collapsed nanotube may be examined with basic energetic considerations. For simplicity, consider

TABLE 1 Parameters of experimentally observed collapsed carbon nanotubes

Tube	Number of walls, n	W_{in} (nm)	W_{out} (nm)	t (nm)	D_{in} (nm)	D_{out} (nm)	Length (μm)
I	9	—	22	6	10.2	16.1	2.4
II	8	15	20	5	9.5	14.8	>1.2
III	7	—	20	4.8	9.7	14.3	>1.9
IV	6	16	20	4	10.2	14.2	>0.2
V	8	16.2	21.5	5.3	10.3	15.6	3

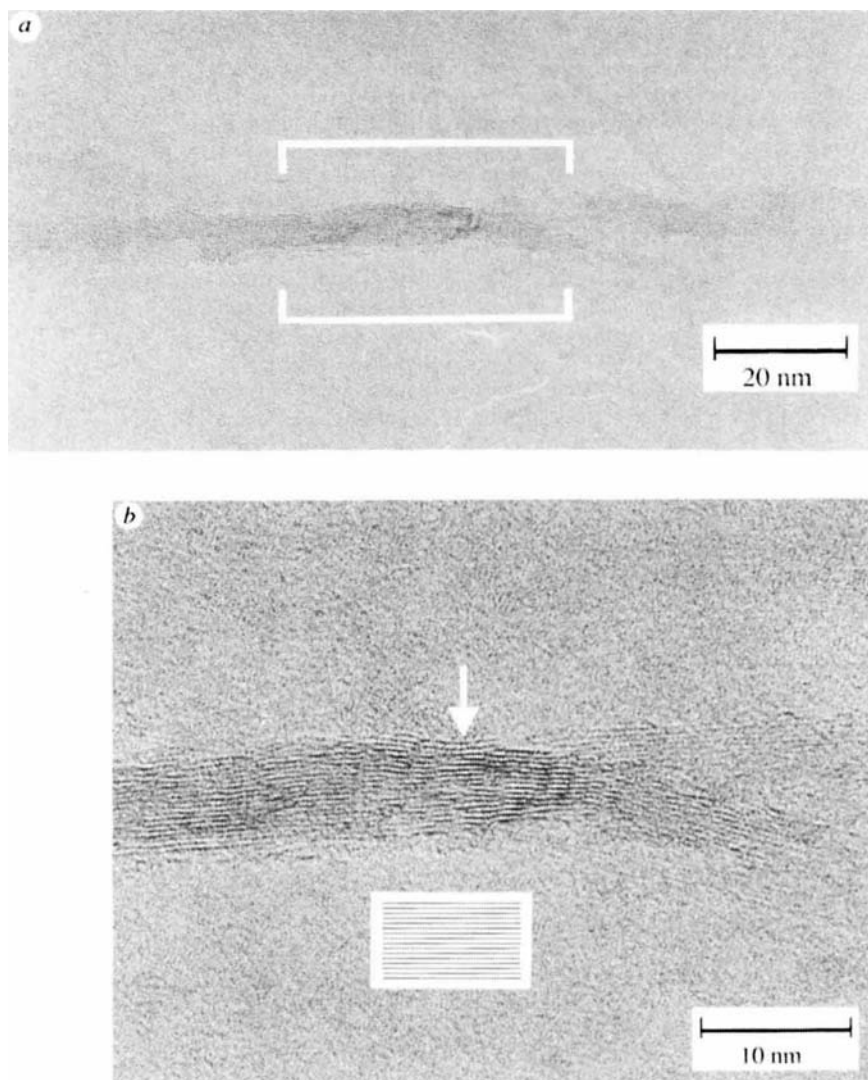


FIG. 3 *a*, High-resolution TEM image of twisted region of a collapsed nanotube. *b*, Close-up of the boxed region in *a*. Inset (not drawn to scale) points to the main feature of the collapsed tube when the flat part is perpendicular to the imaging plane, namely 16 lattice fringes with no gap (region of vertical arrow). It should be noted that details of the fringe structure (not represented in the schematic drawing) are sensitive to the focus condition and orientation of the edges of the collapsed nanotube²⁰.

a single-wall 'inflated' nanotube of radius R . The total energy per atom relative to a flat sheet can be calculated by modelling the tube as a membrane with curvature $1/R$ and curvature modulus k ^{13, 16}. This takes into account the increase in energy due to the introduction of sp^3 character bonding. The energy per

unit length of the tube is then

$$E_{\text{tube}} = k2\pi R/(2R^2) = \pi k/R \quad (1)$$

We obtain excellent quantitative agreement between the predictions of this expression and local density approximation (LDA) calculations of the total energy of carbon nanotubes¹⁷ (for tubes with radii >2.4 Å) when $k = 1.4$ eV.

The flattened (that is, collapsed) version of the same tube is a two-sheeted strip of width slightly less than πR . The curvature is nearly zero everywhere except at the strip edges, where the curvature is very high (see Fig. 4*a*). Assume a radius of curvature at the edges of $r \ll R$, the curvature energy per unit length of this structure is

$$E_c = 2ka/(2r^2) = ka/r^2 \quad (2)$$

where a is the arc length of the curved region on each of the two strip edges. E_c must be greater than E_{tube} from simple geometric considerations. But the flattened tube will also have an attractive van der Waals energy if the thickness of the strip is close to d , the graphite inter-sheet spacing. This attractive energy per unit length, E_v , is proportional to the width of the straight portion:

$$E_v = -E_{\text{vdw}}(\pi R - a) \quad (3)$$

where $E_{\text{vdw}} \sim 0.02$ eV Å⁻² (ref. 18). It is reasonable to assume that r and a are independent of R and only depend on d (for $r, a \ll R$). This means that E_c is a constant. Examining equations 1–3, we see that there must then be a critical value of R such

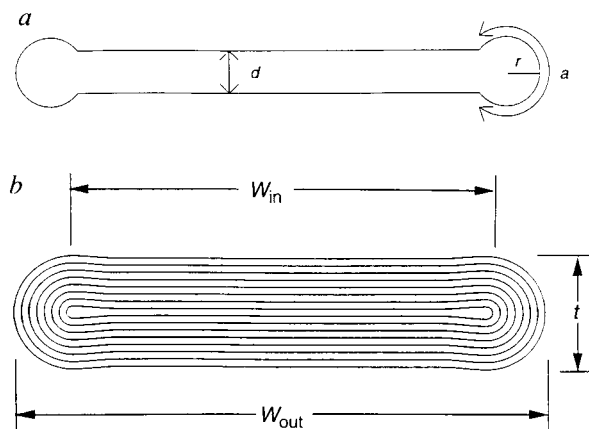


FIG. 4 *a*, Schematic cross-section of a collapsed single-wall tube: d is the interlayer distance, a is the arc length of the curved region, and r is the radius of curvature. *b*, Cross-section of theoretically modelled 8-walled fully collapsed tube with $R = R_{\text{crit}}$.

that for $R < R_{\text{crit}}$, $E_{\text{tube}} < E_c + E_v$ (inflated tube more favourable than collapsed tube), whereas for $R > R_{\text{crit}}$, $E_{\text{tube}} > E_c + E_v$ (collapsed tube more favourable than inflated tube).

Now consider an n -walled tube. Upon flattening, all the walls increase their curvature energy, whereas only the inner one gains attractive van der Waals energy. Thus, $R_{\text{crit}}(n)$ is an increasing function of n . ($R_{\text{crit}}(n)$ is the radius of the innermost wall when inflated). Using a model based on the above considerations, we have estimated (L.X.B. *et al.*, manuscript in preparation) $R_{\text{crit}}(n)$ for several n . We find that $R_{\text{crit}}(1) \sim 8d$, and $R_{\text{crit}}(8) \sim 19d$.

Figure 4b shows our model's predicted cross-section for an $n=8$ fully collapsed tube with $R=R_{\text{crit}}(8)$. For this tube, W_{out} is $72d$ ($=238 \text{ \AA}$), slightly greater than but comparable to $W_{\text{out}} \approx 200 \text{ \AA}$ for the $n=8$ collapsed tube observed experimentally (tubes II and V in Table 1). It must be emphasized that even if the total energy of a flattened tube is greater than that of its inflated counterpart, the flattened tube may still be metastable (that is, not prone to spontaneous 're-inflation'). We estimate that the critical radius for metastability is much less than $R_{\text{crit}}(n)$ for each n (L.X.B. *et al.*, manuscript in preparation).

The observation of stable, flattened nanotubes may have implications for electronic and mechanical nanotube properties. For example, the electronic structure of a collapsed (carbon or other composition) nanotube may differ significantly from that of its cylindrical counterpart. Similarly, the minimum radius of curvature of bending for a flattened tube is expected to be substantially smaller for a flattened tube than for a cylindrical tube.

The interplay between curvature energy and van der Waals energy may also be relevant to the collapse of larger graphitic structures such as carbon whiskers¹⁹. □

Received 27 March; accepted 14 August 1995.

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ACKNOWLEDGEMENTS. We thank F. Ross, C. Nelson and M. O'Keefe of the National Center for Electron Microscopy, Lawrence Berkeley Laboratory, for helpful interactions. This research was supported by the National Science Foundation, and the Director, Office of Energy Research, Office of Basic Energy Services, Materials Science Division of the U.S. Department of Energy. N.G.C. acknowledges support from a Department of Education Graduate Fellowship.

Oxygen isotope evidence against bulk recycled sediment in the mantle sources of Pitcairn Island lavas

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THE hypothesis that subducted sediments survive dehydration and/or melting in subduction zones to become long-lived geochemical reservoirs in the mantle has gained support in recent years^{1–3}. Evidence for such reservoirs is found in the geochemistry of ocean island basalts (OIBs), some of which have isotopic and trace-element characteristics plausibly associated with ancient sedimentary components. In particular, the EM1 mantle end-member has been identified, principally on the basis of strontium, neodymium and lead isotopes, and has been proposed to carry a large sediment fraction^{3–5}. Oxygen isotopes should be sensitive indicators of subducted sediment in the sources of OIBs because minerals that interact with water at low temperatures near the Earth's surface (during weathering, for example) become enriched in ¹⁸O relative to ¹⁶O (ref. 6). We report here the ¹⁸O:¹⁶O ratios of phenocrysts from basalts from Pitcairn Island (southeast Pacific Ocean), which, together with the nearby Pitcairn seamounts, contain among the most extreme EM1 signatures known. We find the oxygen isotope ratios of the phenocrysts to be indistinguishable

from the average for mantle peridotite. These results show that the end-member EM1 signature can be produced in the absence of substantial (>1–2%) recycled sediment in the mantle.

Pitcairn Island basalts were collected on the 1987 *Helios* expedition of the Scripps Institution of Oceanography. Our samples are all basalts with MgO > 4 wt%. Although they were mostly collected as cobbles, they can be confidently associated with the Tedside formation of shield-building Pitcairn lavas on the basis of their chemical and isotopic characteristics (K.A.F. and E.H., unpublished results). The Sr–Nd–Pb isotopic compositions of our samples span a substantial range, up to and including the most extreme EM1 composition documented from Pitcairn Island and seamounts (Table 1 and Fig. 1).

Oxygen isotope ratios were measured at the University of Wisconsin by laser fluorination^{7,8} of olivine, pyroxene and plagioclase phenocrysts. Results for samples and concurrently analysed garnet and olivine standards are presented in Table 1.

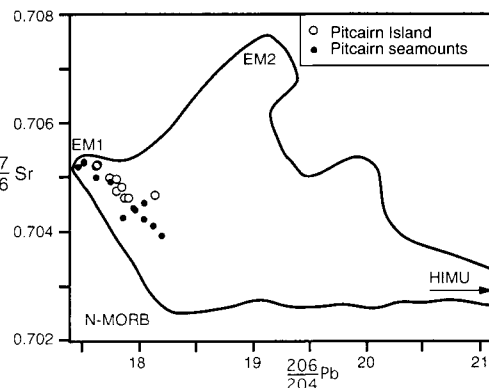


FIG. 1 Sr and Pb isotope ratios of basalts from Pitcairn Island (Tedside volcanics) and the Pitcairn seamounts (Table 1; refs 11, 18, 19). Ranges of ocean island basalts and identified mantle end-members are shown for comparison³. HIMU, N-MORB and EM2 are the radiogenic-Pb-enriched, normal MORB mantle, and enriched-mantle-2 end-members, respectively³.

TABLE 1 Geochemical data for Pitcairn Island samples

Sample	$\delta^{18}\text{O}_{\text{SMOW}}^*$			Whole rock		Olivine ‡	Plagioclase ‡	$^{206}_{204}\text{Pb}$	$^{207}_{204}\text{Pb}$	$^{208}_{204}\text{Pb}$
	Garnet	Olivine	Plagioclase	$\text{SiO}_2^{\dagger}$	$\text{MgO}^{\dagger}$	X_{Fo}	X_{An}			
Standards										
UWG2§	5.77 (5; 0.07)									
KH Ol $_{\text{H}}$		5.32 (2; 0.05)								
SC Ol $_{\text{I}}$		5.25 (7; 0.05)								
NBS-981								16.937	15.492	36.705
Tedside volcanics (Pitcairn Island)										
Pit-16*		5.34	6.09 (2; 0.05)	48.7	4.4	75.7	57.8	18.131	15.515	38.926
Pit-7		5.30 (2; 0.12)	—	48.2	6.4	76.5	58.6	17.833	15.494	38.982
Pit-8		5.19 (2; 0.15)	—	46.3	11.9	82.0	—	17.900	15.494	38.976
Pit-4A		—	6.00	48.9	4.2	—	NA	17.789	15.492	38.970
Pit-11		5.26	—	46.4	12.0	80.6	—	17.858	15.478	38.939
Pit-12		5.11	6.08	49.2	6.3	81.3	62.7	17.789	15.487	38.975
Pit-13		5.09	5.82 (2; 0.00)	47.5	8.1	79.5	72.1	17.735	15.474	38.943
Pit-1		5.22 (4; 0.04)	6.29	48.1	7.1	80.1	NA	17.630	15.460	38.936
Pit-3		5.18 (2; 0.07)	—	48.7	7.8	80.2	—	17.611	15.461	38.912

Pb isotope ratios were measured at DTM by E.H.H. Pb isotopes are reported relative to the given ratios for NBS-981. Sr and Nd isotope ratios on these samples are also typical of the Tedside volcanics and will be reported elsewhere.

* All values are followed by: (number of analyses; standard deviation) if multiple analyses were done.

$$\delta^{18}\text{O}_{\text{SMOW}} = ((^{18}\text{O}/^{16}\text{O}_{\text{sample}} - ^{18}\text{O}/^{16}\text{O}_{\text{SMOW}}) / ^{18}\text{O}/^{16}\text{O}_{\text{SMOW}}) \times 1,000$$

† SiO_2 and MgO values are from analysis of whole rock specimens.

‡ Olivine and plagioclase Fo and An compositions are the averages of multiple analyses taken more than 50 μm from grain edges. All olivine grains contain gradational iron enrichment in the rims, reaching an extreme of ~ 65 . NA, present but not analysed.

§ The nominal value of UWG2 is $5.8 \pm 0.1\text{‰}$.

$_{\text{H}}$ KH Ol, olivine from a xenolith from Kilbourne Hole. SC Ol, olivine from a xenolith from San Carlos.

* $\delta^{18}\text{O}_{\text{epx}} = 5.13$ (2; 0.03).

Multiple analyses were done on half of the samples. Each analysis was from fluorination of 1–5 phenocryst fragments ~ 0.5 –1.0 mm in size, which is also the size of olivine from mantle xenoliths analysed for comparison. The average deviation from the mean of multiple analyses of samples ($\pm 0.07\text{‰}$) is comparable to the analytical precision determined from analysis of standards (± 0.07 to $\pm 0.05\text{‰}$; throughout this paper, all uncertainties are 1σ), suggesting minimal isotopic variability between grains within a given sample. Analysis of standards indicates that the data are accurate to 0.1‰ relative to NBS28 = 9.59‰ on the standard mean ocean water (SMOW) scale⁸.

Oxygen isotope ratios of olivine phenocrysts from eight samples are within analytical uncertainty of each other, averaging $5.21 \pm 0.08\text{‰}$ (Fig. 2). The five plagioclase analyses (four from rocks in which olivine was also analysed) are more variable, with an average of $6.05 \pm 0.15\text{‰}$ (Table 1). The difference in $\delta^{18}\text{O}$ between coexisting plagioclase and olivine in these samples is similar to the expected magmatic fractionation between these phases (~ 0.9 – 1.0‰ for An_{65} at 1,100–1,200 °C; ref. 9), consistent with their being in oxygen isotopic equilibrium at magmatic temperatures. This is an important observation because it provides evidence against the possibility that the olivines are xenocrysts with inherited oxygen isotope ratios unrelated to the host basalts. Petrographic observations and measurements of olivine composition by electron microprobe support this conclusion (Table 1); that is, all eight samples contain Fo_{65-82} olivines, too iron-rich to be mantle-derived xenocrysts ($\sim \text{Fo}_{90}$), but compatible with having crystallized in their host magmas¹⁰.

Submarine glasses from the Pitcairn seamounts have been reported to have high $\delta^{18}\text{O}$ values (up to 6.8–7.4‰), correlated with radiogenic isotope ratios¹¹. Our results on minerals from Pitcairn Island lavas do not show such high $\delta^{18}\text{O}$ values, nor do they vary with radiogenic isotope ratios (Table 1). A significant difference between this study and that of the Pitcairn seamounts is in the phases analysed: we analysed coarse crystals, whereas the analyses of seamount samples were glasses from quenched margins of submarine 'pillows'. There are several advantages to analysing phenocrysts, which we feel outweigh the burden of demonstrating that such crystals are not xenocrystic: (1) crystalline phases, particularly olivine, are much more resistant to iso-

tope exchange with water, so careful hand-picking to exclude grains containing alteration products can filter out isotopic shifts associated with weathering; (2) analysis of a phase common to both basalts and the mantle (for example, magnesian olivine) minimizes the confounding effects of isotope fractionation between sources and derived magmas.

The average $\delta^{18}\text{O}$ value of $5.21 \pm 0.08\text{‰}$ for Pitcairn Island olivine phenocrysts is indistinguishable from the average for olivine from peridotitic xenoliths of mantle peridotite¹² ($5.19 \pm 0.14\text{‰}$) and the average of the two olivine separates from mantle xenoliths analysed in this study ($5.26 \pm 0.05\text{‰}$). These average $\delta^{18}\text{O}$ values are similar to that expected for olivine in the mantle sources of mid-ocean ridge basalt; that is, they are 0.5‰ lower than the average for mid-ocean-ridge basalt (MORB) glasses ($5.7 \pm 0.2\text{‰}$)¹³, which is the fractionation expected between olivine and basaltic liquid as previously documented in experimental and natural studies^{14–16}. The key result of our study is that olivine phenocrysts from end-member EM1 Pitcairn Island basalts are in oxygen isotope equilibrium with plagioclase and indistinguishable at the 0.1‰ level from known upper mantle olivine, as determined directly by studies of peridotite xenoliths and indirectly from studies of MORB glasses.

The $\delta^{18}\text{O}$ values of the phenocrysts in the Pitcairn Island basalts are not consistent with the presence of significant amounts of directly recycled bulk sediment in the EM1 mantle end-member. The $\delta^{18}\text{O}$ value of modern ocean sediment is $\sim 15\text{‰}$ (terrigenous)¹⁷ to $\sim 25\text{‰}$ (pelagic)^{18,19} and that of 'normal' upper mantle olivine is 5.2‰; consequently, for each per cent of recycled sediment added to the mantle, the bulk $\delta^{18}\text{O}$ value of the mixture would increase by ~ 0.1 – 0.2‰ (as would the $\delta^{18}\text{O}$ values of partial melts of these sources). At the 2σ level, the maximum possible deviation between the averages for upper mantle olivines from Matthey *et al.*¹² and Pitcairn Island olivine phenocrysts from this study is 0.06‰ (based on standard deviations of the means), corresponding to a maximum possible sediment contribution to the Pitcairn Island EM1 source of 0.3–0.6%. The most ^{18}O -rich individual olivine analysis from this study is 5.34‰, corresponding to a sediment contribution of 0.75–1.5%. This calculation probably overestimates the sediment

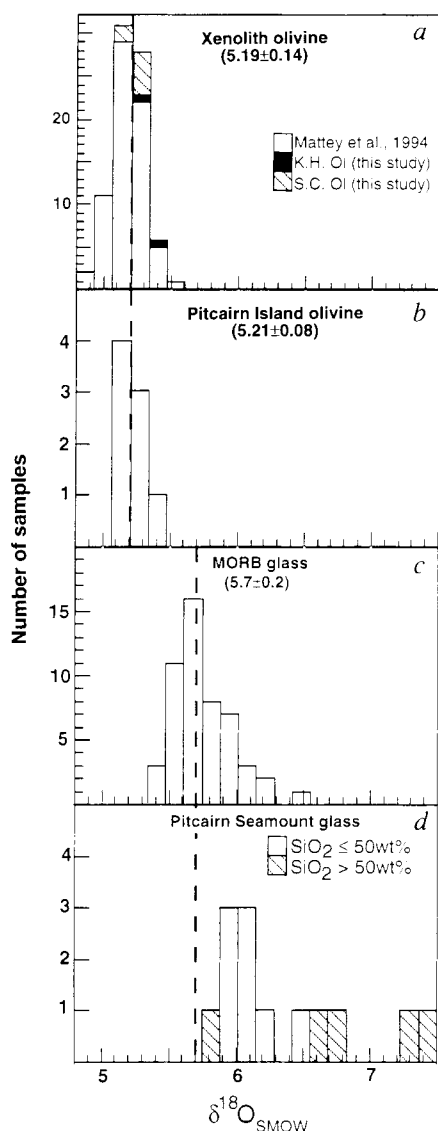


FIG. 2 Histograms of $\delta^{18}\text{O}$ values. *a*, Olivine from mantle xenoliths (ref. 12 and this work); *b*, olivine phenocrysts from Pitcairn Island alkali basalts (this study; each sample is counted only once, so results from multiple analyses of olivines from a single sample have been averaged); *c*, basaltic glasses from a worldwide sampling of MORBs¹³; and *d*, basaltic-to-evolved glasses from the Pitcairn seamounts¹¹.

contribution to the EM1 mantle source of the Pitcairn Island basalts, yet it restricts the amount of sediment to an upper limit of 1.5%. Our data are most consistent with directly recycled sediment being completely absent or present in only trace amounts ($\leq 1\%$).

Although our results and their interpretation are straightforward, the differences between data from this study and results from analysis of Pitcairn seamount glasses¹¹, which suggested 9% sediment in the EM1 source (up to $\sim 20\%$ is permitted for sediment with $\delta^{18}\text{O}$ of 15‰), require explanation. We consider briefly three possibilities:

(1) The $\delta^{18}\text{O}$ values reported from the seamounts are not magmatic values, but instead reflect sample alteration. None of the five samples reported to have $\delta^{18}\text{O}$ values significantly greater than 6‰ was reproduced by replicate analyses in the original report. Subsequent attempts to replicate these analyses have met with mixed results: three of four independent conventional analyses of powders are within 0.2‰ of original reported

values, but the fourth (initially reported as 7.3‰) has yielded markedly different values of 6.5 and 6.0‰ (J. Woodhead, personal communication). Laser fluorination of the freshest glass shards (picked by us and analysed by laser fluorination at the University of Wisconsin) yields MORB-like values ($5.6 \pm 0.1\%$; $n=3$) for samples having reported values of 6.4–6.8‰. Although unusually hydrous glasses might be damaged during pre-fluorination for laser analysis, analysis of other natural basaltic and synthetic glasses by laser fluorination at the University of Wisconsin using the same technique yields values within 0.1‰ of conventional determinations made in other laboratories. These data suggest the possibility that the seamount glasses are heterogeneous in $\delta^{18}\text{O}$. Further work is necessary and should include a demonstration of isotopic equilibrium between glasses and co-existing phenocrysts.

(2) An alternative explanation is that the EM1 component is highly variable in $\delta^{18}\text{O}$ and that the EM1 sources sampled by the Pitcairn Island and Pitcairn seamount lavas differ systematically in $\delta^{18}\text{O}$. Although this cannot be disproved, we consider it implausible given that the source(s) are closely associated spatially and are indistinguishable in their radiogenic isotope and trace-element ratios (Table 1; refs 11, 20, 21).

(3) The final possibility is that the oxygen isotope ratios of the seamount magmas do not represent those of basaltic magmas derived from the EM1 mantle source. Many of the seamount samples are highly evolved. Reported $\delta^{18}\text{O}$ values of glasses and radiogenic isotope ratios are correlated with whole-rock chemistry^{11,20,21}. They define binary arrays between a basaltic end-member having relatively depleted radiogenic isotope ratios and a highly evolved end-member with the EM1 signature. All but one of the seamount samples that have $\delta^{18}\text{O}$ values $\geq 6.5\%$ are SiO_2 -rich: the one sample that is high in $\delta^{18}\text{O}$ and low in SiO_2 is anomalously rich in MgO (14.8%)¹¹ making it likely that the sample is an olivine cumulate and that the glass is considerably higher in SiO_2 than the whole rock. If this chemically evolved end-member were generated at relatively low temperatures (900–950 °C, as could occur either by melting or extensive fractional crystallization of water-rich alkali basalt^{10,22}, or by low-pressure melting of hydrous basalt²³), it could have $\delta^{18}\text{O}$ values in the range of 6.5–7.0‰ (calculated based on data in refs 14–16, 24–26, and M. Palin, personal communication). This estimate seems reasonable, as $\delta^{18}\text{O}$ values in evolved oceanic magmas are often increased by 0.6–1.5‰ above values in related basalts^{24,27–29}. If, as at Pitcairn Island, magmas generated at the Pitcairn seamounts varied with time from EM1-rich to EM1-poor (all with normal $\delta^{18}\text{O}$), one way to explain a compositional array in which an EM1-rich component is preferentially associated with evolved, ^{18}O -rich lavas would be by mixing EM1-poor alkali basalt produced late in the history of the seamount volcanoes with variable proportions of low-temperature partial melts of the pre-existing, EM1-rich volcanic pile. Although the EM1-rich volcanic pile would have normal $\delta^{18}\text{O}$, the partial melts derived from it would plausibly have elevated $\delta^{18}\text{O}$ values, thereby explaining the correlation of an EM1 signature with high $\delta^{18}\text{O}$.

In summary, our results require that the enriched EM1 mantle source sampled by Pitcairn Island basalts contains little, if any, directly added ^{18}O -rich sediment. A sedimentary contribution to the EM1 component could still be invoked if it were added indirectly; for example, if fluid or melt derived from sediment-rich regions of the mantle subsequently metasomatized mantle with normal oxygen isotope ratios, this could produce peridotites with enriched sediment-like incompatible trace element and/or radiogenic isotopic ratios, while leaving the oxygen isotopes little affected. Alternatively, the EM1 signature expressed at Pitcairn Island could be the product of intramantle metasomatism in which sediment was not involved. For example, a metasomatic fluid or melt derived from devolatilization or low-degree melting of mantle peridotite could infiltrate and significantly change the trace-element ratios in other portions of the mantle (lead-

ing to distinctive radiogenic isotope ratios after sufficient time); such a process would have minimal effects on oxygen isotope ratios. □

Received 10 April; accepted 8 August 1995.

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ACKNOWLEDGEMENTS. We thank J. Woodhead for sharing unpublished analyses of seamount glasses, W. White for comments on the manuscript, M. Spicuzza and N. Kitchen for assistance in the stable-isotope laboratory, and P. Carpenter for help with electron microprobe analysis. This research was supported in part by the DOE and the NSF.

Monotreme affinities and low-frequency hearing suggested by multituberculate ear

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MULTITUBERCULATES are an extinct, dentally distinctive group of Mesozoic/early Cenozoic mammals of uncertain affinities¹. We report here the discovery of a multituberculate ectotympanic bone, associated with the malleus in original life position, from two exquisitely preserved auditory regions. This documents, to our knowledge for the first time, incorporation of the angular and prearticular bones (jaw components in non-mammalian tetrapods) into the middle ear of multituberculates, favouring the hypothesized single origin of the ossicular chain in mammals^{2,3}. Morphology and orientation of these elements are strikingly similar to those of the extant egg-laying platypus and echidnas, suggesting a unique common ancestry of these forms⁴, an affiliation once generally discredited^{5–8} but regaining some recent support^{9,10}. The structure of these new multituberculate auditory ossicles, in conjunction with a greatly inflated vestibule and an uncoiled cochlea, implies an ear inefficient for reception of high-frequency airborne vibrations but well suited for bone-conducted hearing.

The triossicular mammalian middle ear transmits vibrations from the tympanic membrane (eardrum), supported by the ectotympanic bone, to the inner ear. Recognition that the mammalian auditory ossicles are transformed lower jaw and jaw suspensory elements of other gnathostomes represents a classic achievement in comparative biology¹¹. The ear bones and the gnathic homologue are the ectotympanic (angular), malleus (articular plus prearticular), incus (quadrate) and stapes (hyomandibular).

The new specimens (V10777.1 and V10777.2, Institute of Vertebrate Paleontology and Paleoanthropology, Beijing) were recovered from the transitional Palaeocene–Eocene Bayan Ulan beds of Inner Mongolia, China. They are assignable to *Lambdopsalis bulla*, a specialized multituberculate, most cranial elements of which, apart from portions of the ossicular apparatus, have previously been described^{5,12}. The new material demonstrates the presence and acoustic role of a well developed and, in every respect, 'typically mammalian' ectotympanic in this multituberculate (Fig. 1). This bone is completely detached from the lower jaw, lies horizontally against the base of the auditory region, and bears an internal groove for attachment of the tympanic membrane. Its position suggests a more medial and horizontal placement of the tympanic membrane than in non-mammalian cynodonts, where the angular bone forms part of the mandible. The body of the *Lambdopsalis* malleus is broad, and bears a long, robust anterior process (Fig. 1). Specimen V10777.2 demonstrates the *Lambdopsalis* malleus to be compound in structure, consisting of an anterior process (goniale, a prearticular derivative) plus the malleus proper (an articular derivative), precisely as in therians and monotremes^{13,14}. The homology of the anterior process of the malleus and the prearticular is affirmed by the presence of a foramen for passage of the chorda tympani (Fig. 2), a branch of the facial nerve¹¹. Detailed descriptions of the new material and a reconstruction of the *Lambdopsalis* middle ear are provided in Figs 1–3.

There is continuing controversy regarding the number of times that the mammalian triossicular system has evolved, recent estimates ranging from once^{2,3,15} to as many as three times^{5,6,16,17}. Of the numerous currently competing hypotheses of multituberculate affinities, four recent cladistic results are summarized in Fig. 4. As indicated below, ossicular evidence decidedly favours the alliance of multituberculate and monotreme, a finding consistent with the suggested single freeing of the ear bones from the dentary during the origin of the mammalian middle ear^{2,3,11}. Otic similarities of *Lambdopsalis* and other mammals include: angular bone detached from the lower jaw, forming an ectotympanic enclosing the fully suspended tympanic membrane; articular and prearticular bones detached from the lower jaw, fusing to form malleus; and mutual spatial relationships between these elements and such basicranial landmarks as the chorda tympani maintained throughout this transformation. Even if the reduction of post-dentary bones in 'advanced' synapsids were prone to convergent evolution, as is often suggested¹⁷, it seems unlikely that this adequately accounts for the numerous intricate resemblances of the mammalian sound-conducting system.

Regarding the relationship of multituberculates to other mammals, the following auditory features of *Lambdopsalis* lend support to the recently revised hypothesis of a monotreme-multituberculate pairing¹⁰. The ectotympanic is horizontally positioned, differing from a relatively vertical angular bone in near outgroups such as *Morganucodon*¹⁸; a horizontal ectotympanic occurring in certain therians is probably convergent¹⁴. The *Lambdopsalis* incus is simple and flat¹², contrasting with its more complicated form in non-mammalian cynodonts¹⁹ and therians^{13,16}. The incus lies dorsal to the malleus as in adult monotremes, a condition strikingly distinct from the anteroposterior relation of the malleus (articular) and incus (quadrate) in non-mammalian cynodonts¹⁹ and therians^{13,14,16}. The ectotympanic contacts the pterygoid anteromedially as in

FIG. 1 a, Ventral view of the left auditory region of *Lambdopsalis* (V10777.3); b, ventral view of partial left ectotympanic and articulated malleus (V10777.1), both in original life position; c, anterior (slightly lateral) view of V.10777.1; and d, dorsal view of a partial right malleus (V10777.2). c, Condyle; ct, foramen for the chorda tympani nerve; e, ectotympanic; er, epitympanic recess; fc, 'fenestra cochleae' (perilymphatic foramen); fi, incudal fossa; fv, fenestra vestibuli; m, malleus; p, pterygoid. V.10777.2-3, from mature individuals, are slightly larger than V.10777.1, from a juvenile having an unerupted last molar (same scale for b-d). The posterior portion of the ectotympanic is missing and a crack separates its anterior and medial portions. The anterior portion of the ectotympanic underlaps the anterior process of the malleus; both bones lie nearly horizontally. The medial portion of the ectotympanic is crescentic and contacts the 'pterygoid' anteriorly. A groove for attachment of the tympanic membrane occupies the bone's inner edge. Unlike the thin, spring-like structure in most therians^{13,16}, the long anterior process of the malleus is robust and extends anteromedially. It is firmly connected, but not fused, to the ectotympanic. The malleus and ectotympanic are loosely attached to the basicranium and probably functioned as a single element acoustically. A foramen for passage of the chorda tympani nerve perforates the anterior process (broken dorsally in V10777.1) roughly midway along its length as in extant mammals^{13,14}. The preserved portion of the transverse part (body) of the malleus bears a broad, concave ventral surface (b); dorsally this region forms a thick, triangular area which rests in the epitympanic recess (c, d). A fossa posterolateral to this thickened area is presumably for the incudal articulation.

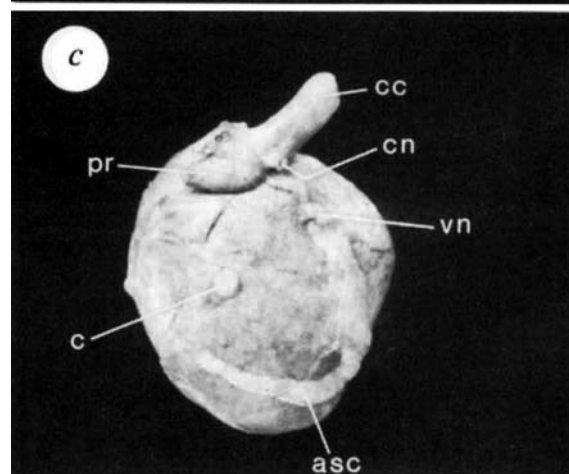
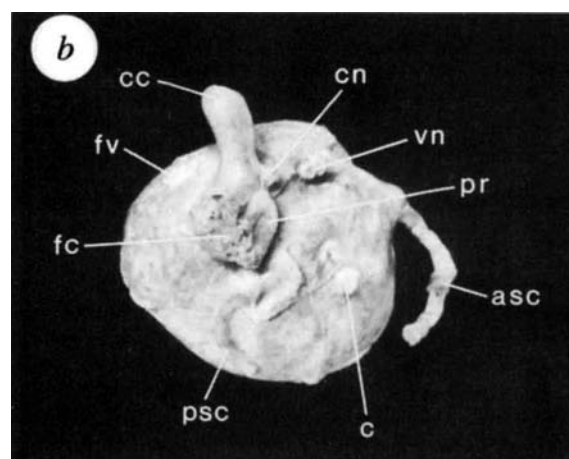
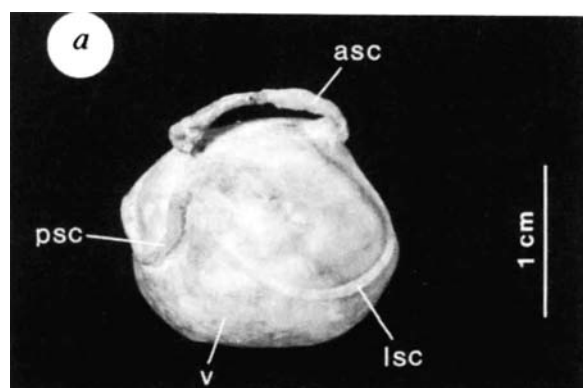
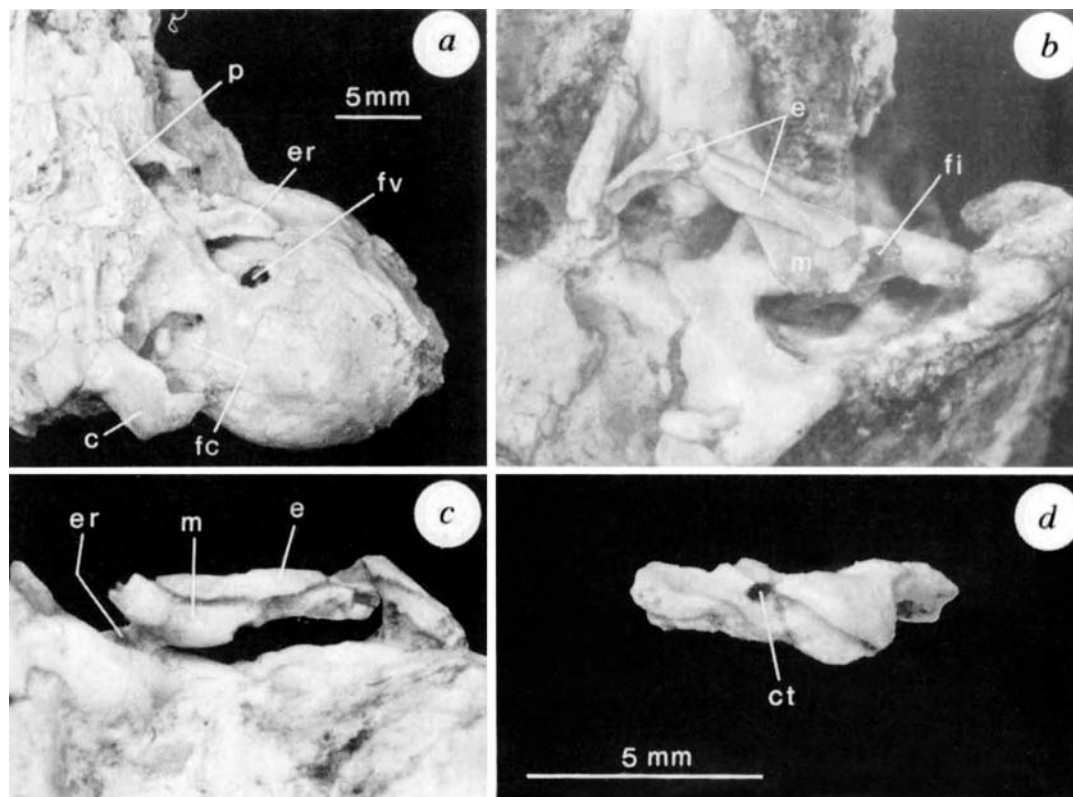


FIG. 2 Natural endocasts of the right inner ear of *Lambdopsalis*. a, Posterodorsal view (V10777.4); b and c, ventral and medial views (V10777.5). Abbreviations as for Fig. 1 plus: asc, anterior semicircular canal; c, crus commune; cc, cochlear canal; cn, cochlear nerve; lsc, lateral semicircular canal; pr, perilymphatic recess; psc, posterior semicircular canal; v, vestibule; vn, vestibular nerve. The semicircular canals are irregular in cross-section whereas the crus commune is circular. The lateral canals and most of the posterior canal are confluent with the vestibular space. The ovoid vestibule has an average volume of 785 mm³ (approximating the vestibule as an ellipsoid), remarkably, more than two orders of magnitude larger than in humans²⁴. The vestibular nerve endocast subdivides, one branch entering the vestibule and the other leading dorsally to the ampulla of the anterior semicircular canal. The cochlear nerve passes the cochlear canal as a single bundle. The cochlear canal is 8.7 mm long from the posterior edge of the 'fenestra cochleae' to its tip. In ventral view its tubular form bends slightly laterally, the basal part being slightly narrower than the anterior part (except for the apical tip). No bony structures are preserved within it. The 'fenestra cochleae' is irregular in shape and is larger than the fenestra ovalis. Measurements (average diameter in mm; followed by number of specimens examined): semicircular canals (0.5 × 1.0;

7); loops of anterior, lateral and posterior semicircular canals (maximum 6.5, 9.1 and 5.2; 7); crus commune (1.1; 5); vestibule (10 × 15; 9); cochlear canal (maximum 1.9; 4); cochlear nerve bundle (0.68; 5); fenestra vestibuli (1.8 × 2.2; 23).

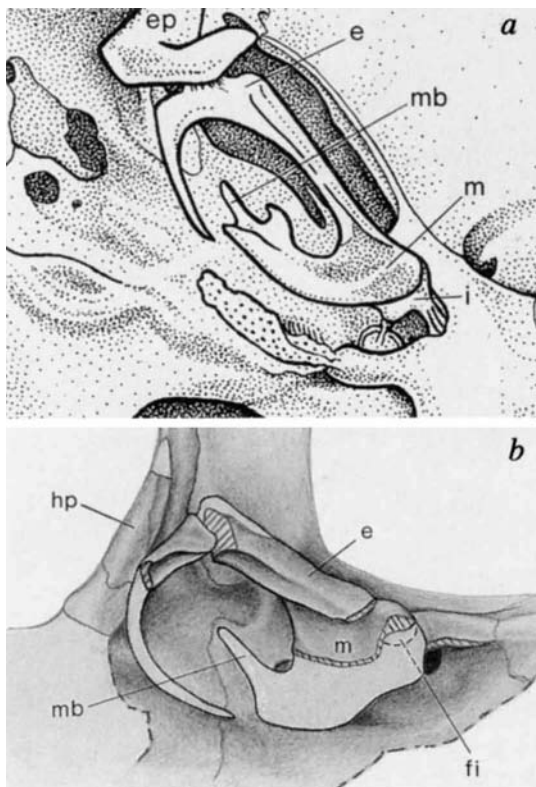


FIG. 3 Middle ears in ventral view of the platypus (a)¹⁰ and *Lambdopsalis* (b) (anterior is up; not to scale). Reconstruction in b is based on specimens described herein and an illustration¹² plus a photograph of V7151.80 from D. Miao. Abbreviations as in Figs 2 and 3 plus: ep, 'ectopterygoid'; i, incus; mb, manubrium. The manubrium and part of the body of the malleus are preserved in V7151.80. Based on the new material reported here, it appears that the unusual posterolaterally directed manubrium of V7151.80 previously figured¹² is due to displacement. The new material thus shows the ossicular arrangement of *Lambdopsalis* to conform closely to the typical mammalian condition, particularly that seen in monotremes, where the manubrium is directed anteromedially. In addition to features mentioned in the text and Fig. 4 (Node 2), the ear of *Lambdopsalis* displays several primitive resemblances to monotremes: the ectotympanic is large and massive relative to, for instance, that in didelphid marsupials; the malleus is large and bears a robust anterior process; the broadly connected malleus and ectotympanic are loosely attached to the basicranium; the malleus lacks a protruding head; and the cochlear canal is uncoiled. The estimated maximum diameters of the tympanum (based on V10777.1, an immature individual) are 5.4×3.2 mm. The calculated primary transformer ratio²³ for transmitting sound vibrations to the inner ear is 3, an extremely low figure. There is no osseous trace of the external auditory meatus in the ear of *Lambdopsalis*, suggesting that it was cartilaginous and that its proximal end extended medially to the ventral side of the promontorium as in monotremes²⁷. This contrasts with the condition in non-mammalian cynodonts where the external auditory meatus would, if present, have been lateral to the angular bone, i.e. lateral to the mandible¹⁵.

monotremes (where it is variously termed 'ectopterygoid' or 'echidna pterygoid').

Many previous assessments of multituberculate relationships have, in our view, been unduly influenced by a reluctance to accept derivation of the peculiar multituberculate molar cusp pattern from a 'reversed triangle' arrangement. Inasmuch as taxa with such bizarrely specialized dentitions as *Shuotherium* are generally accepted as having a 'reversed-triangle' ancestry²⁰, and the multituberculate-like molar pattern of the megachiropteran bat *Harpyionycteris whiteheadi* certainly has a tribosphenic progenitor, the distinctive molar pattern of multituberculates seems

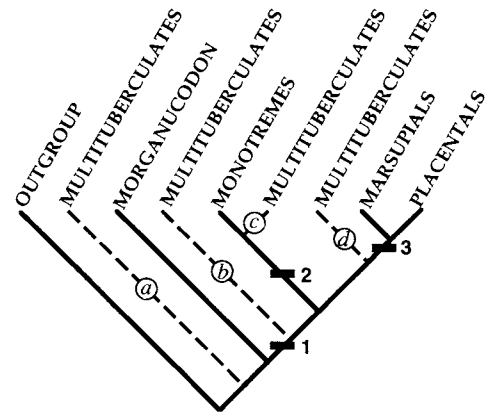


FIG. 4 Summary of previous cladistic proposals (a–d) concerning the phylogenetic affinities of multituberculates. Hypothesis a^{6,28} is consistent with an assumed early divergence for multituberculates, but lacks supporting morphological evidence. Hypothesis d⁷ is based on postcranial and to a lesser extent cranial information; reanalysis of the cranial component of this data set led to proposition of hypothesis b⁸. A recent treatment²⁹ also contradicts hypothesis d. Hypothesis c^{2,4} was founded originally on a shared pattern of the braincase sidewall, a pattern later judged plesiomorphic^{2,15}, and still later as possibly advanced⁹. Hypotheses b, c and d are most parsimoniously interpreted in the context of a single origin of the triosseous system and derivation of the multituberculate molar pattern from a 'reversed triangle' ancestral condition, although these interpretations are sensitive to the currently insecure placement of certain fragmentary fossil forms such as *Kuehneotherium*. With the caveat that more data from other multituberculates is needed, synapomorphies of the auditory region supporting monophyly of indicated groups include: Node 1 (Mammalia⁷): angular detached from mandible, forming ectotympanic; suspension of tympanic membrane by ectotympanic near the basicranial region; medial extension of the external auditory meatus to ventral side of the promontorium; prearticular and articular detached from lower jaw and incorporated into middle ear as the malleus; manubrium mallei present and extends to the centre of tympanic membrane^{15,16}; reduced quadrate (incus) losing the occlusal force from masticatory movement of the mandible^{11,19}. Node 2 (Monotremata + Multituberculata): ectotympanic and malleus horizontally positioned¹⁴; ectotympanic contacts the 'pterygoid'; incus simple and flat¹²; incus dorsal to the malleus. Other features supporting this grouping are listed elsewhere^{9,10}. Node 3 (Theria): anterior process of malleus thinned and reduced; cochlea coiled more than 360°; primary and secondary osseous spiral laminae developed; perilymphatic recess merging with scala tympani; radial pattern of cochlear nerve; and basal extension of basilar membrane³⁰.

no less plausibly derived from a 'reversed triangle' antecedent than from one in which molar cusps were arranged in a longitudinal row. In the absence of dental (or other) evidence convincingly demonstrating a remote phylogenetic placement of multituberculates, or placing some taxon retaining postdentary bones within the clade stemming from the most recent common ancestor of monotremes plus therians, there is little basis for judging the triosseous chain to have originated more than once.

Functionally, the middle ear of *Lambdopsalis* was probably less sensitive to airborne sounds than in modern therians, judging from its close resemblance to monotremes^{21,22}. The estimated area of the tympanic membrane in *Lambdopsalis* is small relative to that of the oval window; the resulting low primary transformer ratio²³(=3) suggests inefficient detection of airborne sounds. An inflated vestibule occurs to varying degrees among many multituberculates and is possibly associated with low-frequency hearing^{5,24}. Increased separation of the stapedial footplate and cochlear base resulting from vestibular inflation dampens sensitivity to high-frequency vibrations given their rapid dissipation with distance from the stapedial footplate²⁵. In addition, vestibular inflation probably enhances bone-conducted hearing; volumetric asymmetry between the scala tympani and

scala vestibuli of the cochlea leads skull-conducted vibrations to produce significant fluid displacement within the cochlea, and hence basilar membrane stimulation²⁵. Bone-conducted hearing is consistent with the fossorial lifestyle postulated for *Lambdopsalis* on the basis of cranial and postcranial evidence^{5,26}. □

Received 3 May; accepted 12 July 1995.

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ACKNOWLEDGEMENTS. We thank E. Allin, J. Hopson, Z. Kielan-Jaworowski, Z. Luo, R. MacPhee, M. McKenna, D. Miao, M. Novacek, R. Presley, J. Rosowski, G. Rougier, N. Simmons, R. Tedford, X. Wang, A. Weil and R. Zhai for instructive comments and discussions. L. Meeker and C. Tarka prepared the illustrations. Work was supported by AMNH, IVPP, Erlan Museum, UCSB and the National Geographic Society.

Cretaceous multituberculate skeleton and the early evolution of the mammalian shoulder girdle

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A Cretaceous eucosmodont multituberculate mammal skeleton has been found in Mongolia with all of the bony elements of the shoulder girdle in place. This specimen demonstrates a different forelimb stance from that recently hypothesized for another Cretaceous eucosmodont. Primitively, it retains a separate ossified interclavicle, as in monotremes and non-mammalian cynodonts. In other respects it shares with therians and their extinct allies key features associated with mobility of the pectoral girdle and shoulder joint during locomotion, and a more parasagittal forelimb posture. This locomotor transformation appears to have evolved just once among the common ancestors of multituberculates and therians, some time before the Late Jurassic.

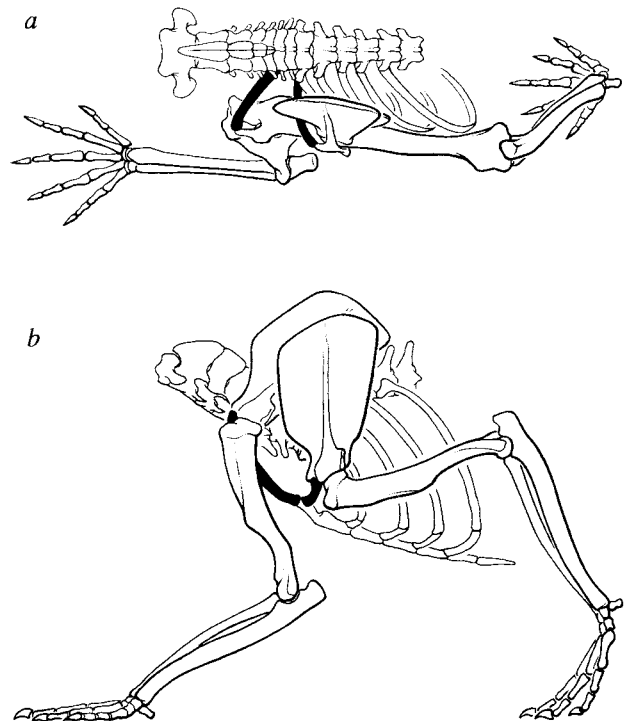


FIG. 1 a, b, Excursion of the left pectoral girdle and forelimb relative to the vertebral column and ribcage in a walking opossum (*Didelphis virginiana*) in dorsal (a) and lateral (b) views¹.

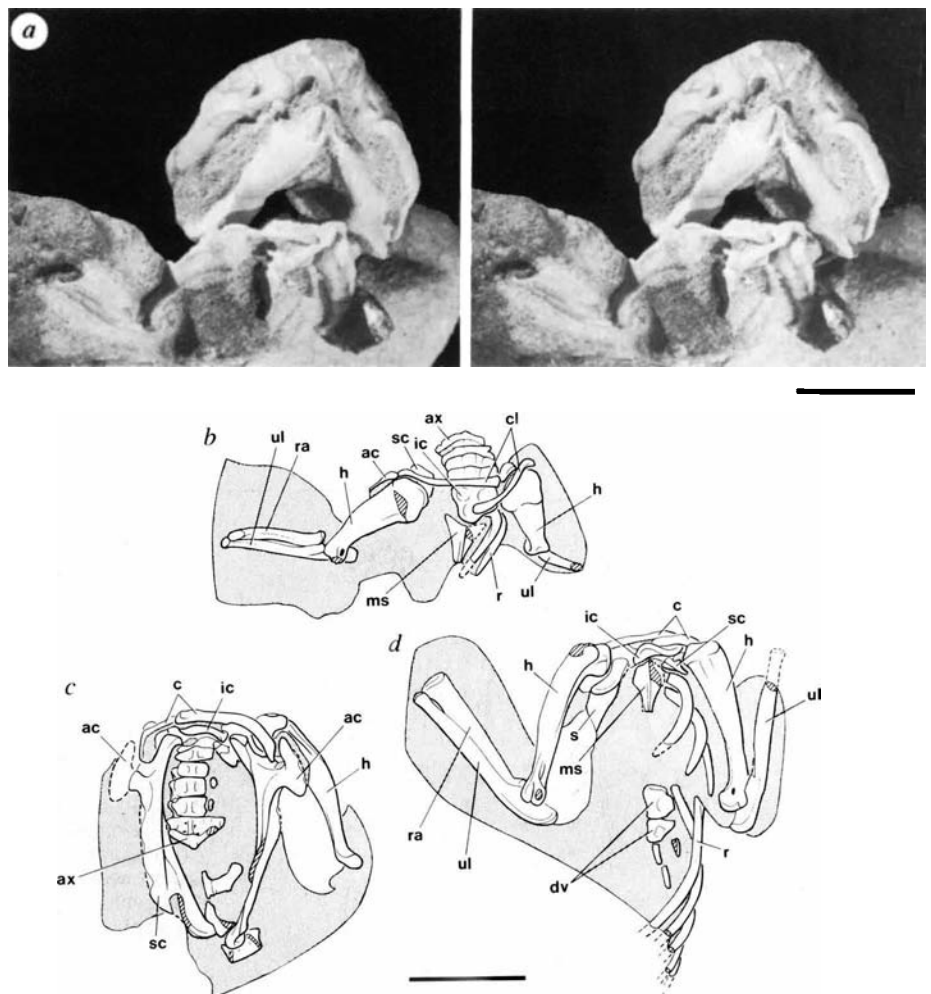
Early in mammalian evolution, locomotor function of the shoulder and forelimb was remodelled^{1–3}. The shoulder girdle began to move with the forelimb during the stride, and the forelimb was repositioned under the shoulder girdle so that the forefoot landed closer to the midline (Fig. 1). During the stride's propulsive phase, shoulder girdle and shoulder joint rotate posteroventrally, with only limited motion (flexion) at the shoulder and elbow joints. Because the elbow joint remains closer to the body wall, its hingelike movements are tightly constrained by a trochlear humero-ulnar joint⁴. This locomotor pattern characterizes most living therian mammals and profoundly influences manoeuvrability and gait⁵.

A more ancient locomotor pattern is present in non-mammalian cynodonts⁶ and primitive Mesozoic mammals⁷, retained in modified form in living monotremes^{8,9}. In the ancient pattern, the shoulder blade is firmly anchored to a median interclavicle or sternum, the socket of the shoulder girdle is broadly exposed in lateral view, the humerus projects away from the body axis at an angle of at least 45°, and the manus is positioned lateral to the shoulder girdle. The humerus generates much of the propulsive component of the stride by retraction and/or long-axis rotation⁸. The elbow joint is not constrained by a trochlear articulation.

Important clues regarding the architecture of the shoulder girdle and forelimb in many Mesozoic mammals have been discovered over the years^{10–16}, and the fate of bones that were reduced or eliminated in the course of this functional reorganization has been traced^{17,18}. Nonetheless, the stance of anterior limbs has been hypothesized from incomplete specimens to conform with the ancient pattern¹⁶.

A skeleton of a Mongolian Cretaceous multituberculate, PSS-MAE-103 [type Djadokhta Formation (Campanian), from 'ruins locality', Bayn Dzak], preserves the entire pectoral girdle, manubrium sterni and proximal segments of the forelimb (Fig. 2). It clearly pertains to a eucosmodontine taeniolauidoid multituberculate, provisionally referred to *Bulganbaatar nemeghtaataroides*¹⁹ (Fig. 2a). Clavicle and interclavicle (not

FIG. 2 PSS-MAE-103 (Paleontology Section, Mongolian Academy of Sciences), cf. *Bulganbaatar nemegtbaataroides*. **a**, Stereo figure of the articulated skull and anterior part of the skeleton in anterior view; **b–d**, anterior (**b**), dorsal (**c**) and ventral (**d**) views of the anterior part of the skeleton. Identification is based upon proportionately long premaxillary postero-lateral process, nasal–parietal contact, U-shaped frontoparietal suture, anterior orbital shelf, rounded zygomatic arch with lateral crest, single ventrolaterally positioned infraorbital foramen, palatal vacuities, absence of palatal thickenings, deep jugular fossa, and advanced M¹ cusp formula. Abbreviations: ac, acromion; ax, axis; c, clavicle; co, coracoid; dc, deltopectoral crest; dv, dorsal vertebrae; ec, ectepicondyle; en, entepicondyle; enf, entepicondylar foramen; gl, glenoid; gr, coracoid groove; gt, greater tuberosity; h, humerus; hh, humeral head; ic, interclavicle; isf, incipient supraspinous fossa; lt, lesser tuberosity; ms, manubrium sterni; pco, procoracoid; pcof, procoracoid foramen; pp, posterior process of coracoid; r, rib; ra, radius; sc, scapulocoracoid; sf, supraspinous fossa; ss, scapular spine; ssp, secondary scapular spine; uc, ulnar condyle; ul, ulna. Scale bars, 1 cm.



represented in previously described Mongolian Cretaceous multituberculates¹⁶), and scapulocoracoid are present. The long scapular blade is deeply concave laterally, as in previously reported multituberculate scapulae^{14,16}, and has an elongate acromial process. Scapula and coracoid fuse relatively early in development in multituberculates. There is no trace of a scapulocoracoid suture in PSS-MAE-103 or other undamaged specimens^{10,16}. The multituberculate coracoid forms only the ventral tip of the glenoid fossa. Unlike other mammals, there is no development of a coracoid posterior process (Fig. 3*a, c*). The slender, curved clavicle is strap-shaped throughout most of its length but becomes rod-shaped near its articulation with the cloverleaf-shaped interclavicle, which is arched anteroposteriorly (Fig. 3*a*). The interclavicle has shallow anterior fossae for articulation with the clavicles. It has a short ventral process that rests on a keeled manubrium sterni (Fig. 3*b*). The proximal end of the humerus is dominated by a bulbous head, with rounded tuberosities on its anterior margin (Fig. 3*d, e*). As in other multituberculate humeri^{12,14,16,20}, the distal end has a broad intercondylar groove, prominent ulnar condyle, and moderate-sized epicondyles. There is less humeral shaft torsion (approximately 15°) (Fig. 3*f*) than in the basal mammals (*Morganucodon*)⁷ or monotremes⁹. Forearm bones equal the humerus and scapula in length (Fig. 3; Table 1).

A separate ossified interclavicle in multituberculates is a primitive feature shared with non-mammalian cynodonts and monotremes^{6,10,17,21}. In therian mammals, in contrast, the dermal portion of the interclavicle never appears in development, and the endochondral portion fuses with the manubrium sterni^{17,18}.

In other respects the multituberculate pectoral girdle is structurally advanced^{10,20}, resembling that in therians (Fig. 4) and not so abducted as hypothesized recently¹⁶.

TABLE 1 Measurements of PSS-MAE-103, cf. *Bulganbaatar nemegtbaataroides*

	Size (mm)
Skull	
Length	26.5
Scapulocoracoid	
Length	17.5
Length of glenoid	4.4
Width of glenoid	1.4
Length of acromion	5.2
Width of blade	(3.0)
Clavicle	
Length	9.0
Mid-shaft, minimum width	0.5
Mid-shaft, width	0.9
Medial end, diameter	1.0
Interclavicle	
Depth	3.3
Width	4.3
Manubrium sterni	
Length	5.4
Width	(5.0)
Humerus	
Length	15.7
Maximum diameter of head	3.0
Width of distal end	3.5
Radius	
Length	12.0
Width of distal end	2.5
Ulna	
Length	16.0
Width of distal end	1.2

Length, width and depth are maximum values unless stated otherwise. Parentheses indicate estimation.

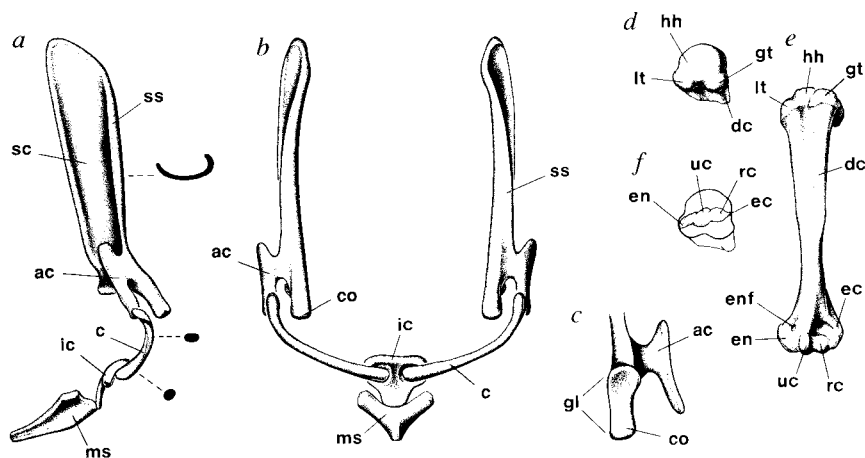


FIG. 3 Scaled reconstruction of the pectoral girdle, manubrium sterni and humerus of PSS-MAE-103, cf. *Bulganbaatar nemegtbaataroides*. a, b, Pectoral girdle and manubrium sterni, in right lateral (a) and anterior (b) views with cross-sections of the scapular blade and clavicle; c, right glenoid and acromion in posteroventral view (enlarged 20% compared with other figures); d, e, left humerus in proximal (d), and anterior (e) views; f, left humerus in proximal view showing orientation of distal end. See Fig. 2 for abbreviations.

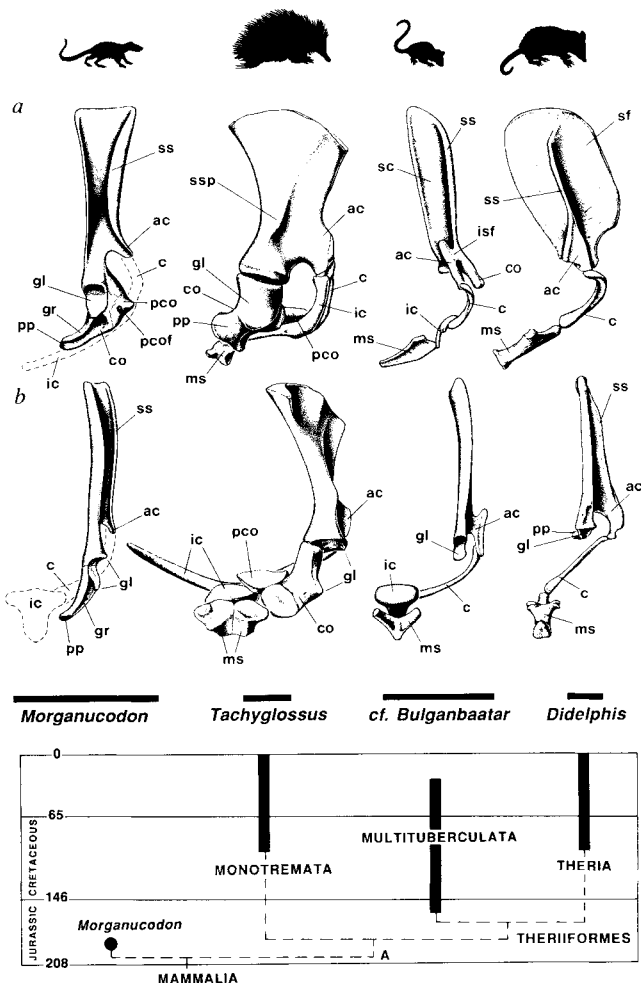


FIG. 4 Early evolution of the mammalian pectoral girdle as shown by comparison of an early mammal (*Morganucodon*), monotreme (subadult *Tachyglossus* with open scapulocoracoid and intrasternal sutures), multituberculate (cf. *Bulganbaatar*), and therian (*Didelphis*) in lateral (a) and posterior (b) views. See Fig. 2 for abbreviations. Scale bars, 1 cm. Solid bars show known stratigraphic range. Apomorphies (22) in the pectoral girdle are listed below, using *Morganucodon* and tritylodonts³⁰ as successive outgroups. Node A: scapulocoracoid suture fused; procoracoid foramen absent; coracoid groove absent. Theriiformes²⁵: acromion and scapular spine positioned lateral to the glenoid (=incipient supraspinous fossa); coracoid posterior process reduced (equal to, or shorter than, length of coracoid glenoid); glenoid width reduced relative to humeral head; coracoid glenoid faces posteroventrally; coracoid glenoid concave (rather than concavoconvex); procoracoid very reduced, lost or fused with manubrium sterni; clavicle medial end rod-shaped. Theria: crescentic supraspinous fossa along entire length of scapular blade; acromion recurved to parallel scapular blade; separate interclavicle absent (=claviculosternal contact present). Monotremata: coracosternal contact; coracoid and procoracoid overlap interclavicle; clavicle and interclavicle fused; interclavicle lateral process long, contacting acromion; interclavicle posterior process fan-shaped. Multituberculata: coracoid glenoid subrectangular (rather than piriform); coracoid posterior process absent; interclavicle cloverleaf-shaped; interclavicle arched anteroposteriorly.

clavicle from the anterior margin of the shoulder blade so it can function as a mobile strut with a pivot at the sternum. In non-cursorial therians, the clavicle follows an angular excursion of as much as 40° during a fast walk^{1,2} (Fig. 1).

Additional features are present that in therians appear to increase the range of motion at the shoulder joint¹. These include the concave surface of the coracoid portion of the glenoid (not saddle-shaped as in non-mammalian cynodonts⁶, basal mammals⁷ and monotremes^{3,6}) and the reduction in transverse (or anteroposterior) width of the glenoid socket to about one-half the diameter of the humeral head (Table 1). The shoulder joint's ball-and-socket architecture and narrow glenoid in multituberculates and therians may allow a greater range of humeral movements¹².

The humerus and elbow joint were positioned closer to the body wall than observed in monotremes^{8,9} or reconstructed in non-mammalian cynodonts⁶ or basal mammals⁷. This is indicated by marked ventral (not lateral) orientation of the glenoid, reduction in size of humeral epicondyles, and hingelike form of the elbow joint (suggested by prominent, narrow ulnar condyle and broad intercondylar groove on the distal end of the humerus, approaching the form of the therian trochlear joint⁴) (Fig. 3e).

Controversy surrounding phylogenetic relationships among multituberculates, monotremes and therians^{23–25} is partly due to incomplete fossil remains of Mesozoic multituberculates²⁶ and monotremes and extreme transformation of skull and dentition in Cenozoic monotremes²⁷. Few cranial or dental characters

Several features are present in PSS-MAE-103 that in therians increase the mobility of the shoulder girdle¹. These include reduction of the procoracoid and the posterior process of the coracoid, posteroventral position of the acromion adjacent to the shoulder joint, and mobile clavicle–interclavicle joint. The procoracoid and coracoid, reduced in early development in therians^{18,22}, are not present as separate ossifications in adult multituberculates. Interpretation of clavicle–interclavicle joint mobility is based on smooth, rod-shaped medial ends of the clavicles (not striated and flattened as they are in non-mammalian cynodonts^{6,14}) and open clavicular depression on the interclavicle. Together, these structural features disassociate the

support alternative resolutions. Traditionally, multituberculates have been grouped with monotremes and various extinct Mesozoic mammals as 'prototherians', based on interpretations of the bony composition of the side wall of the braincase. However, homology of sidewall ossification²⁸ and its presence in multituberculates²⁹ have recently been challenged. Here we suggest that persuasive character evidence is provided by the postcranial skeleton^{3,25}. Six characters from the pectoral girdle and forelimb support a closer relationship among multituberculates, therians and their allies (*Gobiconodon*¹³ and the dryolestid *Henkelotherium*¹⁵) than between any of these taxa and monotremes (Fig. 4). Parallel evolution of therian-like shoulder girdles of mammals cannot be invoked until one or more of the aforementioned taxa that exhibit an advanced condition are linked cladistically to taxa, such as monotremes, that lack such features. We suggest that a mobile pectoral girdle and shoulder joint and a forelimb posture with the elbow near the body wall arose only once, some time before the Late Jurassic, in a common ancestor of multituberculates, therians and their extinct allies. □

Received 12 January; accepted 20 July 1995.

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ACKNOWLEDGEMENTS. We thank C. Abraczinskas for the finished illustrations; W. Amaral for preparation of the specimen; and A. Biewener, M. Carrano, J. Hopson, F. Jenkins Jr, Z. Kielan-Jaworowska, H. Larsson, J. Meng, R. Presley, H.-D. Sues, J. Wible and J. Wilson for review of the manuscript. The research was supported by the National Geographic Society, the NSF, and The David and Lucile Packard Foundation (to P.C.S.).

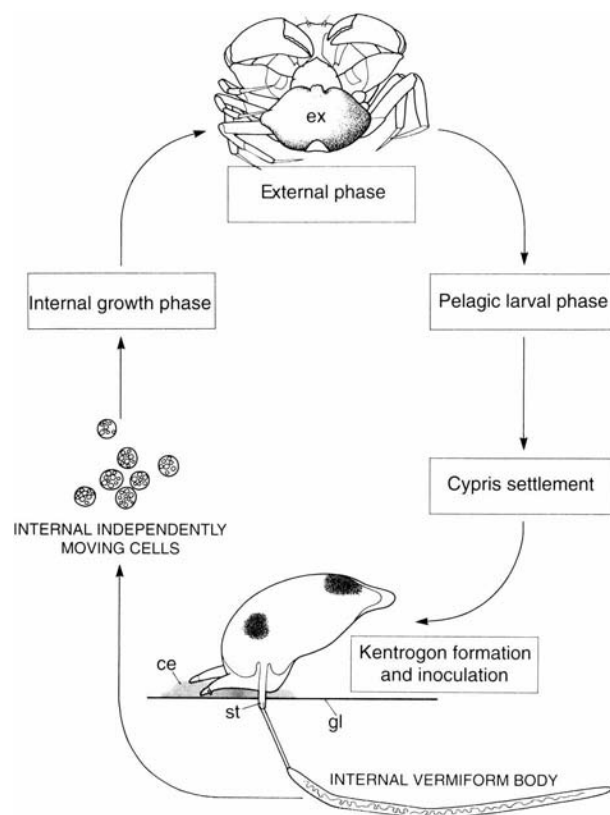
A new motile, multicellular stage involved in host invasion by parasitic barnacles (Rhizocephala)

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RHIZOCEPHALANS are barnacles (Cirripedia), but are extremely specialized for parasitic life on decapod crustaceans. A cypris larva settles and develops into a new instar, the kentrogon, which inoculates the host with the parasite. The very early primordial parasite has been argued to consist solely of embryonic stem cells or even eggs¹, but the true nature of this unknown stage has remained a puzzle for more than a century². We present data from *in vitro* experiments on the rhizocephalan *Loxothylacus panopaei* documenting that, unlike previous postulations, the recently injected parasite is not naked embryonic cells, but has the form of a motile, vermiform body, enclosed in an acellular sheath. After a period of maturation the vermiform body splits up into a number of naked and independently moving cells, which in our *in vitro* experiments disperse by amoeboid movements. This suggests that, *in vivo*, the cells disperse in the haemolymph of the host crab, where each has the potential to develop into an adult parasite, although in most cases only one will succeed.

FIG. 1 The generalized life cycle of *Loxothylacus panopaei*. Attached beneath the abdomen of the host crab, the external parasite (externa, ex), up to 1 cm wide, releases broods of nauplius larvae. The lecithotrophic larvae pass through four nauplius stages until they finally reach the cypris stage (200 µm long). The cypris, a larval type unique for cirripedes, terminates the pelagic phase by settling on the gill of a prospective host. A kentrogon (150 µm long) develops underneath the cuticle of the settled cypris. The kentrogon develops a hollow cuticular stylet (st), that penetrates the cuticle of the gill (gl). The stylet then



serves as a hypodermic needle to inoculate a motile, vermiform body. A period of vigorous motions results in the detachment of the vermiform body from the stylet. After less than 24 h the motions slow down and the core of the vermiform body begins to split up into independently moving single cells. The sheath of the vermiform body eventually ruptures and the parasite cells escape to the surroundings. The fate of these cells is not known but it seems likely that each has the potential to develop into an adult organism, although only one will normally succeed. ce, Cement. (Drawn by Beth Beyerholm.)

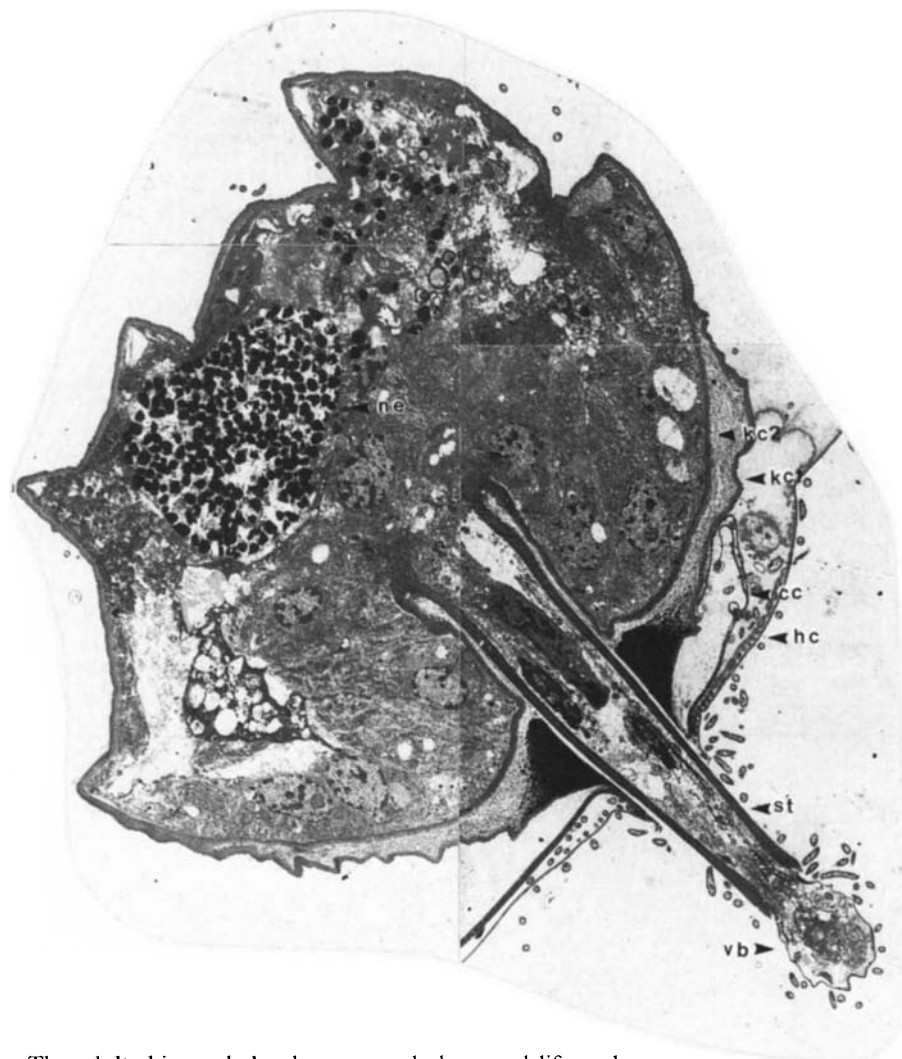


FIG. 2 TEM mosaic micrograph of a transverse section through a kentrogon caught in the process of inoculating the parasitic material through the cuticle of the host and into the haemocoel. Four layers of cuticle are discernible. The thin cuticle of the gill of the host (hc), the cuticle of the cypris carapace (cc) and two cuticle layers of the kentrogon (kc1, kc2). Underneath the first-formed kentrogon cuticle layer (kc1) develops a second layer (kc2) owing to a partial moult (note that cuticle layers kc1 and kc2 fuse). The stylet (st) is part of the second kentrogon cuticle (kc2). During penetration it passes through the first kentrogon cuticle (kc1), the cypris cuticle (cc), and the cuticle of the crab, thereby reaching the blood space of the gill where it expels the primordial parasite in the form of a vermiform body (vb). Note the presence of an eye (ne), which was transferred during metamorphosis from the cypris to the kentrogon.

The adult rhizocephalan has a morphology and life cycle so specialized to parasitism that it shares few characters with other arthropods. In fact, the Rhizocephala can be recognized as Cirripedia, and even as Crustacea, only by means of their larval stages and their spermatozoa^{3,4}. Although ontogeny in the Rhizocephala closely resembles that seen in other cirripedes up to settlement of the cypris larvae, events in the ensuing metamorphosis take a very different course. In other cirripedes the settled cypris develops into a suspension-feeding barnacle, but the rhizocephalan cypris moults to the highly specialized kentrogon, which uses an injection stylet to penetrate the host's integument^{1,5}. Thereafter follows a void in our knowledge, because the nature of the parasitic material injected into the host has not been identified until a small, but differentiated, growth stage appears adjacent to the ventral nerve cord (far removed from the initial site of penetration)⁶. During the endoparasitic growth phase the parasite infiltrates the host with trophic rootlets. Eventually the parasite emerges under the abdomen as an almost structureless reproductive body called the externa⁴.

We initiated the present study to elucidate the nature of the parasitic material inoculated into the host through the stylet. As object of study we chose the rhizocephalan *Loxothylacus panopaei* (Sacculinidae) parasitizing the mud crab *Rhithropanopeus harrisii* (Xanthidae), a well-known host-parasite system very suitable for laboratory maintenance⁷.

Unlike the 'classical rhizocephalan' *Sacculina carcini*, which settles on the limbs of the host crab⁸, the cypris of *L. panopaei* settles in the branchial chamber. Within 24 hours of attachment to a gill, the cypris metamorphoses into the kentrogon, and within another 24 hours the kentrogon has formed a cuticular stylet. Finally, at about 60–72 hours after attachment, the stylet

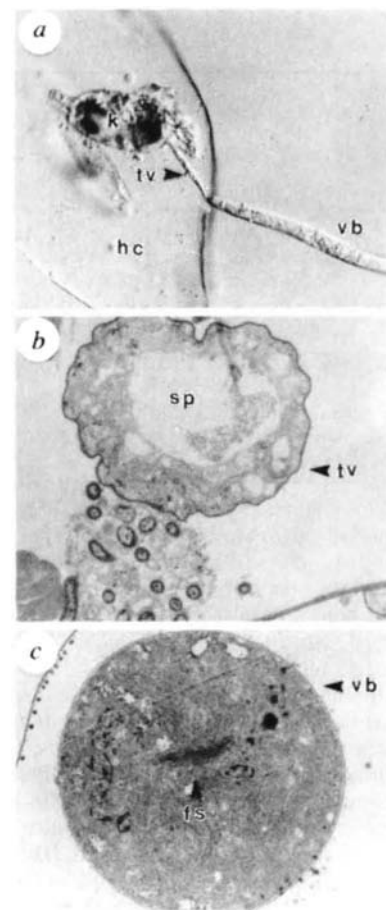


FIG. 3 Differential interference contrast micrographs. a, A kentrogon (k) attached to the gill cuticle of the host (hc). The kentrogon has recently discharged the vermiform body, separated into two distinct parts: the vermiform body itself (vb) connected to the stylet (not shown) by a short and much narrower, hollow tube (tv). b, TEM micrograph of a cross-section through the short, hollow tube (tv), where a large empty space (sp) occupies the centre. c, TEM micrograph of a cross-section through the vermiform body (vb): a fibrous structure (fs), located near the centre of the body, represents the zigzag-shaped organ in Fig. 4a; an acellular matrix that envelops the vermiform body can be seen as a thin, dark layer in b and c.

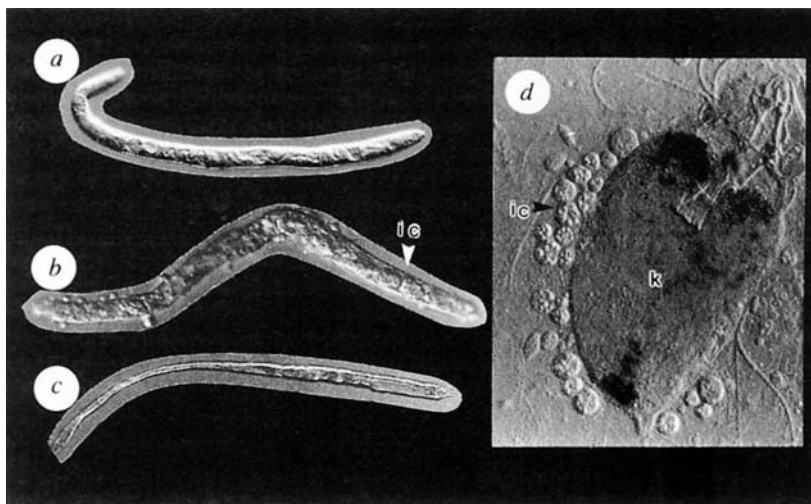


FIG. 4 Differential interference contrast micrographs. *a*, The vermiform body shortly after inoculation; the distinct zigzag-shaped structure extending through the core of the vermiform body is surmised to be involved in the vigorous movements seen shortly after inoculation (see Fig. 3c). *b*, The vermiform body ~16 h after inoculation. The motions have stopped, the zigzag line has disappeared, and the core has split up into single, motile cells (ic). *c*, The empty acellular sheath of the vermiform body ~2 d after inoculation; at this time the motile cells shown in *b* have escaped to the exterior through a rupture. *d*, The individual, motile parasitic cells (ic) liberated from the acellular vermiform body sheath (see *c*) to seawater (*in vitro* experiment) and surrounding a developing kentrogon larva (*k*). Note the identical morphology of these cells and those enclosed within the vermiform body in *b*. *In vivo*, the parasitic cells would be situated in the blood space of the host and travel away from the gill.

penetrates through the integument of the crab and discharges a large vermiform body into the haemolymph of the host in one continuous process lasting about 2 minutes (Figs 1, 2 and 3a). The vermiform body is the first, previously unknown, stage of the internal parasite. It measures 400 μm by 10 μm and is enclosed within a very delicate, 30 nm thick, acellular sheath. The sheath did not react when stained for chitin with colloidal gold labelled with wheatgerm agglutinin and viewed by transmission electron microscope (TEM), whereas the gill cuticle of the crab reacted positively. Although negative, this result does not contradict the proposal that the sheath represents an epicuticle, as that which occurs in the trichogon larva involved in the metamorphosis of rhizocephalan males. Within the sheath, the vermiform body contains nothing but relatively large cells of uniform size and morphology and a zigzag-like structure, which runs through the core of the entire vermiform body. Immediately after inoculation, the vermiform body remains at rest and connected to the tip of the stylet by a hollow tube 30–40 μm long, continuous with, but smaller in diameter than, the sheath of the body itself (Fig. 3a, b). After about 30 minutes the vermiform body begins slow sinuous bending and peristaltic movements running from the distal free end to the hollow tube still connecting it with the stylet. Analysis by TEM revealed that the zigzag-shaped organ consists of fibrous material surrounded by a membrane (Fig. 3c). We suggest that this central structure is involved in the coordinated movements, which within the next few hours result in detachment from the hollow tube, allowing the vermiform bag to move freely in the surrounding liquid. In our *in vitro* experiments the liquid consisted of isotonic sea water. Experiments using detached, but otherwise intact, gills revealed that the vermiform bodies may migrate from the infection site, one of the lateral branches of the phyllobranchiate gill, to the main gill axis. The migration period is restricted to an 8–16-hour period after which the undulating motions of the vermiform body slow down and its morphology starts to change. The zigzag-shaped structure disappears and the cells within the sheath split up into about 25 individual cells, 10 μm in diameter and rotating about their own axis. Eventually the sheath ruptures, releasing cells that spread through the surrounding fluid by alternating flexure and rotating movements (Fig. 4).

Because rhizocephalans have a highly specialized morphology, the nature of their common ancestor can be reconstructed only by comparison with closely related taxa. Recent data from larval ultrastructure and sequencing of ribosomal DNA suggest that the most recent common ancestor to all Cirripedia had already evolved the suspension-feeding lifeform seen in most extant barnacles^{9,10}. However, the evolutionary transition from such a suspension-feeding crustacean to a parasite lacking gut, limbs, segmentation and almost any trait disclosing an arthropod an-

stry, is not easy to envisage. Our study does not solve how this evolutionary transition occurred. But the demonstration that the first endoparasitic stage is a multicellular and motile body, perhaps representing a cuticle-covered instar, differs radically from all previous explanations and could indicate that the morphological shift from external larva to internal parasite was not as abrupt as previously thought^{1,11,12}.

We consider the rhizocephalan kentrogon to be homologous to the juvenile barnacle, which emerges after shedding of the cypris carapace^{5,13}. But whereas the juvenile barnacle remains attached to the substratum, the vermiform body resulting from the rhizocephalan kentrogon can move freely in the surrounding fluid. After settlement on a parasite externa, rhizocephalan males metamorphose into an elongate stage armed with setae, the so-called trichogon, which subsequently migrates through the brood chamber and is implanted as a hyperparasitic dwarf male¹⁴. It remains obscure, however, whether any homology exists between the trichogon and the vermiform stage described here.

The observation that the early, vermiform endoparasite subsequently splits up into single, naked and independently moving cells is a unique event for the Rhizocephala and also without any obvious equivalent in other parasitic arthropods^{12,15}. Judging from the autonomous behaviour of the freed cells in isotonic seawater, it is very unlikely that they should again aggregate to form the later interna. Instead we believe that each has the potential to develop into an individual parasite. This could explain many of those incidences of multiple infections where multiple external parasites are not interconnected by a common root system⁴.

The rhizocephalan ontogeny comprises an exceptional series of developmental events. Notable are: (1) the metamorphosis from a cypris (not discernible from those seen in ordinary barnacles) by way of the kentrogon and an internal vermiform body to a single naked cell within the host; (2) the ensuing development leading from one such cell to a differentiated and cuticle-clad parasite; and (3) that the developing endoparasite defeats the humoral and cellular defences of its host and rapidly takes control of its physiology, morphology and behaviour^{16,18}. The ease with which the vermiform endoparasite and the subsequent free-moving cells are handled in the laboratory offer promising prospects for future research, and we believe that this system deserves attention from more than a narrow group of carcinologists. □

Received 5 June; accepted 21 July 1995.

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ACKNOWLEDGEMENTS. We thank D. Rittschof for hospitality during a research visit to the Duke University Marine Laboratory, T. Olesen for technical assistance, B. Neuhaus for help with gold labelling, and J. Lützen for advice and discussions. This work was supported by grants from the University of Copenhagen (to H.G.) and the Danish National Research Council (to J.T.H.).

A genetic basis for familial aggregation in multiple sclerosis

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GENETIC–environmental interactions probably underlie spontaneous human autoimmune disorders, a category of complex traits thought to include multiple sclerosis (MS)¹. The geographical distribution and familial aggregation of this disease have often been ascribed to the role of infectious agents², but there is no consensus. Increased family risks range from 300-fold^{3,4} for monozygotic twins to 20–40-fold⁵ for biological first-degree relatives over the general population prevalence of 0.1% (ref. 6). We screened a population-based sample of 15,000 individuals with MS by using standardized, personally administered questionnaires to identify adopted index cases and/or those who had adopted relatives. The frequency of MS among first-degree non-biological relatives living with the index case was no greater than expected from Canadian population prevalence data and significantly less than for biological relatives. These findings indicate that familial aggregation of MS is genetically determined: no effect of shared environment was detectable.

Population-based studies of common spontaneous human autoimmune disorders have shown that recurrence risks for monozygotic twins range from 20 to 30% (refs 3, 4, 7–9) and are several-fold greater than for first-degree relatives. These findings strongly suggest the operation of genetic factors. However, the increased degree of environmental sharing by monozygotic versus dizygotic twins and by siblings versus other relatives leads to ambiguity.

Studies of adopted children require large populations and patient pools for which ascertainment is relatively free of bias. They have been used with success in behavioural disorders to discriminate between genes and environment^{10,11} but have not been reported in autoimmune disorders. This is probably because these disorders are less common and affected individuals who are adopted are rare.

MS has a population prevalence of 0.1% and a lifetime prevalence of 0.2% among north Europeans living in high-risk areas such as Canada⁶. Familial aggregation is well documented¹. Increased familial risks range from 30% for monozygotic twins^{3,4} to 3–4% for first-degree relatives^{1,5}. If familial aggregation of

TABLE 1 Kaplan–Meier lifetime risks for biological relatives of 815 multiple sclerosis index cases

Biological relative	Number	Number with MS	Age-adjusted risk $\pm$ s.e. (%)
Parents	1,581	30	2.0 $\pm$ 0.35
Siblings	2,138	53	3.6 $\pm$ 0.50
Children	1,336	9	2.7 $\pm$ 1.09

See Kaplan and Meier¹². Data updated from ref. 12.

MS were solely determined by genes, the risk for non-biological relatives would be as for the general population. If it were determined by familial environment, risks would be as for biological relatives sharing the same environment. An intermediate risk would suggest a joint contribution by genes and familial environment.

Fifteen thousand well-characterized, longitudinally followed patients were surveyed from 14 regionally based MS clinics across Canada. Each person was asked whether he/she was adopted and/or had non-biological first-degree relatives. This study included (1) adopted index cases, their adopting parents, adoptive siblings and adopted children and (2) non-adopted index cases, their adopted siblings and adopted children. The date of 'adoption' was defined as when the adoptee began to live uninterruptedly with the adoptive family.

Across centres, family history data were collected systematically from one or more informed family members, usually the index case, and verified according to the protocol outlined in previously published work on familial risk data^{1,5}.

Two hundred and thirty-eight MS adoptees who lived with their adopting parents by the age of one were identified. They had a total of 470 adopting parents (6 were single-parent adoptions), 155 adoptive siblings and 13 adopted children. Non-adopted MS index cases had a total of 563 non-biological relatives (190 siblings; 373 children). MS adoptees who came into the adoptive family after the age of one were excluded. Individuals 'adopted' by a biological relative lacked discriminating power and were excluded, as were non-Caucasian non-biological adoptive relatives, given the low rate of MS in non-Caucasians⁶.

For comparison with the non-biological relatives, we used Kaplan–Meier¹² age-specific risk curves specific for biological relatives (parents, siblings and children separately) of index cases⁵ (see Table 1). These curves were applied to each category of non-biological first-degree relative. For each non-biological relative, the age-specific expected risk for MS was calculated, correcting for the 'remaining risk' after the age at the time of this study (or death, if applicable). Individual risks were summed for each category of non-biological relative. The probability of the observed number affected (or fewer), given the expected number affected, was based on a Poisson distribution¹³. For these analyses, males and females were combined because of the small number of non-biological relatives. Males and females were equally represented in each 'at risk' group.

As Table 2 shows, the observation of only a single affected non-biological first-degree relative was significantly less than

TABLE 2 Expected versus observed number of non-biological relatives with multiple sclerosis

Non-biological relatives	No. of multiple sclerosis cases		
	Age-adjusted Expected	Observed	Poisson probability ¹⁴ (P)
Parents (N = 470)	9.2	1	0.0010
Siblings (N = 345)	10.7	0	2.3 $\times$ 10 ⁻⁵
Children (N = 386)	5.5	0	0.0041
Total (N = 1201)	25.4	1	2.5 $\times$ 10 ⁻¹⁰

that predicted from the biological, age-adjusted risk data ($P=2.5 \times 10^{-10}$). This is not different from the expected 1.2 affected individuals among 1,201 assuming no familial environmental effect (based on the general population prevalence of 0.1%; ref. 6).

Considering risk for parents separately before and after adoption of the index case, no non-biological parent developed MS after adopting an index case (the age-adjusted expected number of cases was 4.5). One adopting parent had diagnosed MS before the adoption (age-adjusted expected number, 4.7). Not unexpectedly, the mean age of biological parents at the birth of the index case (28 years) was significantly less than the mean age of parents at the time of adoption (33 years). No cases of MS were identified among non-biological siblings and children.

Among 238 adopted index cases, 34 had information on 46 biological parents (4 affected with MS) and 59 had information on 127 biological siblings (1 affected). Although ascertainment was certainly biased and incomplete, these rates are substantially greater than the population prevalence, but comparable to those for first-degree biological relatives⁵.

Data for control diseases in the adopted and adoptive relatives of MS index cases (not shown) indicate that the low rate of MS found is not explained by a general lack of awareness of medical disorders affecting non-biological first-degree relatives.

The observation that the prevalence of MS among non-biological first-degree relatives of MS index cases is similar to that in the general population challenges the notion of a transmissible agent or any familial microenvironmental factor accounting for familial aggregation. We interpret these findings to indicate that familial aggregation in MS is genetically determined. This implies that pedigrees of single-case and multi-case families can be used to develop genetic models of inheritance.

It would not be surprising if these findings were to prove generalizable to other autoimmune disorders. They may serve to divert the search for environmental clues away from specific uncommon viruses to a broader category of events occurring early in life that are applicable to regional populations, for example the multiple factors found to strongly influence penetrance of spontaneous autoimmunity in inbred strains of non-obese diabetic mice¹⁴. □

Received 11 April; accepted 14 July 1995.

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ACKNOWLEDGEMENTS. This research was funded by the Multiple Sclerosis Society of Canada Scientific Research Foundation. We thank the following MS Clinic/Site Study Coordinators for data collection: C. Harris, N. Cheyne, M. Hader, B. Davis, A. Royal, M. Perera, M. Penman, K. Stevenson, L. Murray, G. Smith, C. Edgar, J. Haynes, D. Southwell, R. Arnaoutelis, C. Laforce, C. Masse, P. Weldon, K. Taylor and G. Alcock; we also thank H. Armstrong and J. Smith (London), and R. Farquhar, L. Mashal, I. Yee, T. Hicks, D. Dymet and N. Greig (Vancouver) for assistance. Additional members of the Canadian Collaborative Study Group are: D. Bulman, G.P.A. Rice (London, Ontario); S. A. Hashimoto, D. Paty, J. J. F. Oger (Vancouver, British Columbia); L. Metz, R. Bell (Calgary, Alberta); S. Warren (Edmonton, Alberta); W. Hader (Saskatoon, Saskatchewan); T. Auty, A. Nath (Winnipeg, Manitoba); T. Gray, P. O'Connor (Toronto, Ontario); R. Nelson, M. Freedman (Ottawa, Ontario); D. Brunet (Kingston, Ontario); R. Paulseth (Hamilton, Ontario); G. Francis (Montreal Neurological Institute, Montreal, Quebec); P. Duquette (Hopital Notre Dame, Montreal, Quebec); T. J. Murray, V. Bahn (Halifax, Nova Scotia); and W. Pryse-Phillips (St John's, Newfoundland). Joan Marshall indirectly suggested this study.

Cardiac and adipose tissue abnormalities but not diabetes in mice deficient in GLUT4

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THE insulin-sensitive glucose transporter, GLUT4, is the most abundant facilitative glucose transporter in muscle and adipose tissue, the major sites for postprandial glucose disposal. To assess the role of GLUT4 in glucose homeostasis, we have disrupted the murine GLUT4 gene. Because GLUT4 has been shown to be dysregulated in pathological states such as diabetes and obesity, it was expected that genetic ablation of GLUT4 would result in abnormal glucose homeostasis. The mice deficient in GLUT4 (*GLUT4*-null) are growth-retarded and exhibit decreased longevity associated with cardiac hypertrophy and severely reduced adipose tissue deposits. Blood glucose levels in female *GLUT4*-null mice are not significantly elevated in either the fasting or fed state; in contrast, male *GLUT4*-null mice have moderately reduced glycaemias in the fasted state and increased glycaemias in the fed state. However, both female and male *GLUT4*-null mice exhibit postprandial hyperinsulinaemia, indicating possible insulin resistance. Increased expression of other glucose transporters is observed in the liver (GLUT2) and heart (GLUT1) but not skeletal muscle. Oral glucose tolerance tests show that both female and

male *GLUT4*-null mice clear glucose as efficiently as controls, but insulin tolerance tests indicate that these mice are less sensitive to insulin action. The *GLUT4*-null mice demonstrate that functional GLUT4 protein is not required for maintaining nearly normal glycaemia but that GLUT4 is absolutely essential for sustained growth, normal cellular glucose and fat metabolism, and expected longevity.

Normal glucose homeostasis is the result of a balance between the appearance of glucose in the blood and its cellular uptake and use¹. These processes are controlled by the action of hormones (that is, insulin and glucagon) and by a family of membrane proteins through which glucose facilitatively enters tissues^{2,3}. The function of these glucose transport proteins (GLUTs) is modified in derangements of glucose homeostasis such as diabetes and obesity^{4,5}. GLUT4 is unique in that it is expressed as the major transporter only in tissues in which glucose uptake is stimulated by insulin (that is, adipose cells, cardiac and skeletal muscle)^{6,7}. Insulin stimulates glucose transport by eliciting the translocation of an intracellular pool of GLUT4 to the plasma membrane⁸. The disruption of the GLUT4 gene was undertaken to determine the developmental and metabolic consequences of abolishing the most abundant glucose transporter in insulin target tissues.

To obtain embryonic stem (ES) cells containing a disrupted gene for GLUT4, pGLUT4-KO1 (Fig. 1a) was made linear and electroporated into WW6 ES cells⁹. DNA from neomycin-resistant clones was subjected to Southern blot analysis and hybridized to DNA fragments outside (probe B) and inside (probe A, results not shown) pGLUT4-KO1 to detect homologous recombinants (Fig. 1a). Two ES cell lines, 6 and 22, were obtained that had one disrupted allele of the gene for GLUT4 (Fig. 1b). This 'positive-negative' selection strategy (neomycin and gan-cyclovir, respectively; Fig. 1b) resulted in a GLUT4-specific targeting frequency of 5%.

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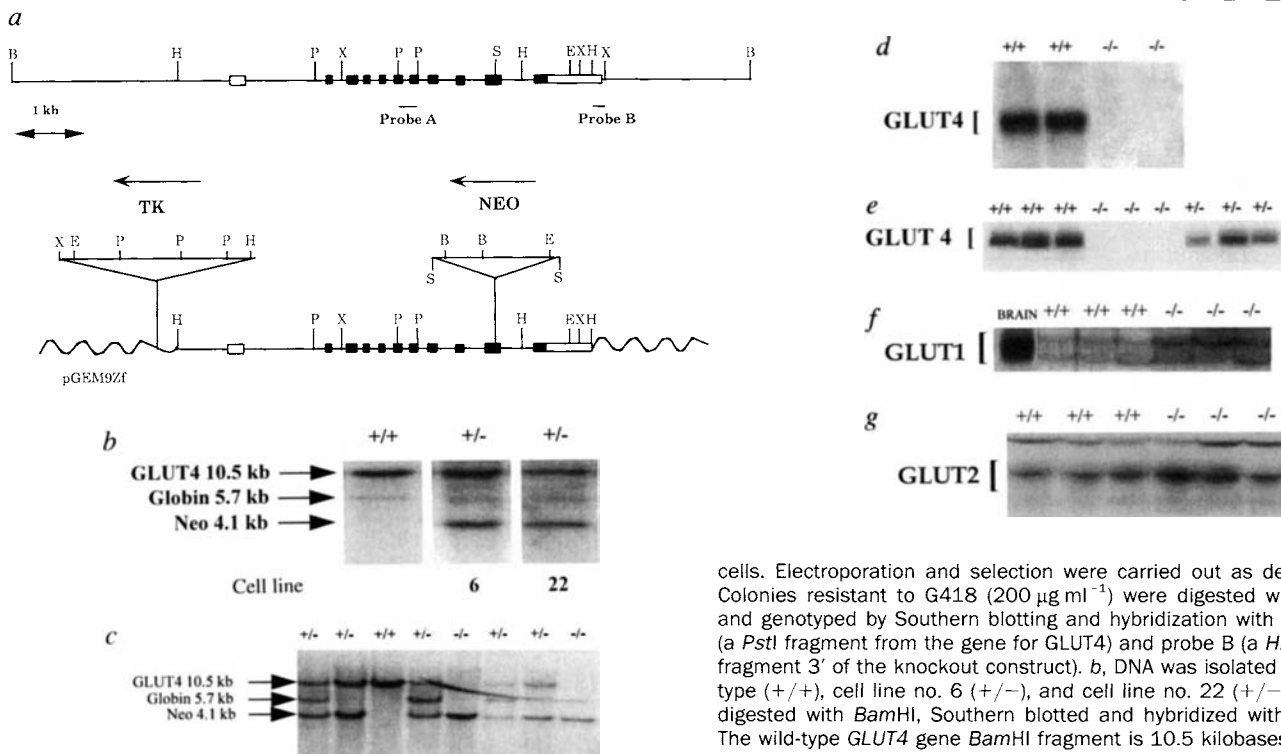


FIG. 1 Disruption of the locus of the gene for GLUT4. **a**, Restriction map of the murine gene for GLUT4 (top) and a neomycin targeting vector (bottom). Open rectangles, non-coding exons; solid rectangles, coding sequences. Restriction endonuclease recognition sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sac*I; X, *Xba*I. **b**, Southern blot analysis of DNA from wild-type (+/+), heterozygous (+/-) GLUT4 knockout ES cell lines. **c**, Southern blot analysis of DNA from a line no. 6 heterozygote (+/-) mating resulting in wild-type (+/+), heterozygous (+/-) and homozygous (-/-) GLUT4-knockout offspring. **d**, Northern blot analysis of RNA from hindlimb skeletal muscle demonstrating a lack of GLUT4 mRNA in (-/-) GLUT4-null mice. **e**, Western blot analysis of hindlimb skeletal muscle demonstrating a lack of GLUT4 protein in (-/-) GLUT4-null mice. **f**, Western blot analysis of total heart membranes showing a 1.5-fold increase in GLUT1 in (-/-) GLUT4-null mice over controls. **g**, Western blot analysis of liver homogenates demonstrating a 1.7-fold increase in GLUT2 in the liver of homozygous (-/-) GLUT4-null mice over controls.

METHODS. **a**, The murine GLUT4 gene and promoter were cloned by plaque hybridization to the rat GLUT4 complementary DNA (6) from a genomic 129/Sv λ library. The gene for neomycin resistance (NEO) under the control of the phosphoglycerate kinase (PGK) promoter was inserted into the unique *Sac*I site of exon 10 of the gene for GLUT4 in a direction opposite to the transcription of GLUT4 for positive selection. The gene for thymidine kinase (TK) under the control of the PGK promoter was inserted into the pGEM9Zf plasmid backbone for negative selection. The resultant plasmid, pGLUT4-KO1, was linearized in a 5' polylinker *Sfi*I site and mixed with a suspension of 2×10^7 WW6 ES

cells. Electroporation and selection were carried out as described¹⁸. Colonies resistant to G418 ($200 \mu\text{g ml}^{-1}$) were digested with *Bam*HI and genotyped by Southern blotting and hybridization with a probe A (a *Pst*I fragment from the gene for GLUT4) and probe B (a *Hind*III-*Xba*I fragment 3' of the knockout construct). **b**, DNA was isolated from wild-type (+/+), cell line no. 6 (+/-), and cell line no. 22 (+/-) (ref. 19), digested with *Bam*HI, Southern blotted and hybridized with probe B. The wild-type GLUT4 gene *Bam*HI fragment is 10.5 kilobases (kb). The homologous recombination of GLUT4-KO1 gives a 4.1-kb *Bam*HI fragment. Additionally, a 5.3-kb *Bam*HI fragment is detected, corresponding to 1,000 copies of a β -globin transgene incorporated into the WW6 genome (telomere of chromosome 3), which is universally detectable^{9,20}. Chimaeric mice were generated¹⁸ and germline transmission was confirmed by Southern blotting. **c**, F₁ heterozygotes (+/-) from cell lines no. 6 and no. 22 were mated to produce homozygotes (-/-). Genotyping was carried out as in **b**. **d**, Total RNA was extracted from 100 mg of hindlimb skeletal muscle from 7-week-old females with TriReagent (Molecular Research) and 25 μg was resolved on a 1.2% formaldehyde-agarose gel and blotted onto nylon membranes. Membranes were hybridized with the 2.6-kb *Eco*RI insert from the rat GLUT4 gene cDNA⁶ and the image was resolved with a phosphorimager (Molecular Dynamics). The data shown are representative of 5 animals of each genotype. **e**, Skeletal muscle homogenates (50 μg per lane) were subjected to SDS-PAGE and transferred to nitrocellulose. After confirmation of uniformity of protein loading and transfer, membranes were immunoreacted with an antiserum against the carboxy terminus of GLUT4 (1:1,000)²¹ and revealed with the Enhanced Chemiluminescence Reagent (Amersham) with HRP-coupled goat anti-rabbit IgG (1:3,500, Southern Biotechnology). Data are representative of 6 animals of each genotype. **f**, Total crude membranes were purified from homogenates of the heart²² and immunoblotted (100 μg per lane) as detailed in **e**. A C-terminus antibody to GLUT1 (ref. 21) was used (1:200) and 2 μg of brain homogenate was included as a control. **g**, Liver homogenates (50 μg per lane) were subjected to western blot analysis as described in **e**. A C-terminus antibody to GLUT2 (residues 497-509) was used (1:200).

Blood glucose, insulin and glucagon levels were determined in female and male GLUT4-null mice in the fasted and fed states to assess the *in vivo* effect on glucose homeostasis of abolishing the main route of insulin-stimulated glucose entry into the

muscle and adipose tissue. GLUT4-null male mice do not control their blood glucose as successfully as female GLUT4-null mice, showing a 34% decrease in fasting and a 20% increase in fed glycaemias compared with normal controls (Table 1). The fasted and fed glycaemias in GLUT4 males do not show a diabetic phenotype but do show that there is a sexually dimorphic response to the lack of GLUT4. Surprisingly, blood glucose levels in GLUT4-null females were not significantly elevated in either the fasted or fed states (Table 1). Fasting insulin levels were unaltered in GLUT4-null mice; however, the fed insulin levels were approximately 5-6 times that seen in control mice (Table 1). Glucagon levels in GLUT4-null mice were not significantly different from controls (Table 1).

One way for the GLUT4-null mouse to compensate for the lack of GLUT4 and maintain normal glycaemia would be the overexpression of other glucose transporters in insulin-

TABLE 1 Serum metabolites and hormone levels in 2–4-month-old *GLUT4*-null and control mice

	Females		Males	
	Control	<i>GLUT4</i> -null	Control	<i>GLUT4</i> -null
Glucose (mg l ⁻¹)				
Fasted	708 ± 27 (14)	743 ± 48 (12)	937 ± 40* (15)	615 ± 43 (13)
Fed	1,206 ± 51 (8)	1,187 ± 45 (11)	1,342 ± 61§ (9)	1,617 ± 89 (7)
Insulin (ng ml ⁻¹)				
Fasted	2.3 ± 0.3 (3)	3.4 ± 0.7 (7)	0.76 ± 0.16 (6)	0.41 ± 0.18 (4)
Fed	3.8 ± 0.8* (3)	24.3 ± 3.0 (6)	7.4 ± 0.16* (3)	35.3 ± 3.4 (3)
Glucagon (pg ml ⁻¹)				
Fasted	76.15 ± 1.5 (5)	66.4 ± 7.2 (4)	59.3 ± 12.6 (3)	52.2 ± 4.1 (5)
Fed	82.7 ± 23.8 (4)	52.5 ± 3.9 (6)	58.8 ± 2.8 (5)	47.6 ± 10.7 (3)
Lactate (mmol l ⁻¹)				
Fasted	4.1 ± 1.3 (4)	1.8 ± 0.2 (6)	3.2 ± 0.2 (4)	2.3 ± 0.4 (6)
Fed	9.5 ± 1.9‡ (4)	2.5 ± 0.3 (6)	6.2 ± 0.2* (4)	2.9 ± 0.2 (6)
FFA (μequiv. l ⁻¹)				
Fasted	2,432.3 ± 309.9 (4)	1,723.8 ± 273.0 (6)	2,726.6 ± 176.5 (4)	2,442.8 ± 318.4 (6)
Fed	1,368.7 ± 360.2 (4)	918.7 ± 101.5 (6)	1,288.4 ± 156.5† (4)	573.8 ± 29.98 (6)
β-Hydroxybutyrate (mg l ⁻¹)				
Fasted	409 ± 42† (4)	43 ± 18 (6)	251 ± 46* (4)	30 ± 11 (6)
Fed	0.26 ± 0.06 (5)	0.26 ± 0.03 (4)	0.29 ± 0.02 (5)	0.26 ± 0.01 (3)

Statistical analysis was performed with a two-tailed unpaired Student's *t*-test. All *P* values are from comparisons between *GLUT4*-null mice and wild-type control mice. Values are represented as means ± s.e.m. Significance was accepted at *P* ≤ 0.05. Numbers in parentheses are numbers of individuals. Whole blood (150–300 μl) was drawn from the orbital sinus for serum metabolite analysis by using heparinized microcapillary tubes. After withdrawal, blood was centrifuged immediately, the serum removed, placed in a fresh tube containing 10 ng aprotinin and frozen immediately on dry ice. Blood from fasted animals was collected after a 16-h fast and blood from *ad libitum*-fed animals was drawn beginning at 1.30 a.m. Blood glucose levels were measured with a One Touch II monitor (Lifescan) on whole blood or by the glucose oxidase method (Sigma) on serum. Commercial radioimmunoassay kits (Linco) containing rat insulin or glucagon antibodies were used to measure serum insulin and glucagon levels. Serum levels of lactate and β-hydroxybutyrate were measured with colorimetric kits (Sigma). Serum levels of non-esterified FFAs were measured colorimetrically with a kit from Amano.

* *P* ≤ 0.001; † *P* ≤ 0.005; ‡ *P* ≤ 0.01; § *P* ≤ 0.05.

responsive tissues. *GLUT1*, the other glucose transporter found in skeletal muscle and fat and the major glucose transporter of the brain, was not upregulated in these tissues (results not shown). *GLUT1* protein was overexpressed by 1.5-fold in the heart compared with normal controls (Fig. 1*f*). The overexpression of *GLUT1* in the heart is unlikely to contribute to maintaining a normal postprandial glycaemia because the heart constitutes a small percentage of the body's insulin responsive tissue. *GLUT2*, the low-affinity glucose transporter, is overexpressed 1.7-fold in the liver of *GLUT4*-null mice (Fig. 1*g*). Overexpression of *GLUT2* in the liver is also seen in metabolic states (that is, starvation and diabetes) where glucose is being made by the liver^{10,11}. The role of liver *GLUT2* overexpression in maintaining normal glycaemia in *GLUT4*-null mice remains to be determined. Finally, the levels of *GLUT3* and *GLUT5* in total hindlimb skeletal muscle were measured by western and northern blot analyses, respectively. Neither transporter was found to be more abundant in *GLUT4*-null mice than in normal controls (results not shown). Because overexpression of the known glucose transporters does not seem to be responsible for maintaining normal glycaemia in *GLUT4*-null mice, the possibility exists that another insulin-responsive glucose transport system has been activated in the muscle.

To assess the response of *GLUT4*-null mice to a bolus of glucose, oral glucose tolerance tests (OGTTs) were performed (Fig. 2*a*). The results of these tests indicated that after the administration of glucose, female *GLUT4*-null mice had significantly higher glycaemias than the normal controls. Male *GLUT4*-null mice had higher initial glycaemias but these were not significantly different from the normal controls. Eventually, both female and male *GLUT4*-null mice cleared glucose as efficiently as the normal controls, but demonstrated a decreased sensitivity to insulin during an insulin tolerance test (ITT) (Fig. 2*b*). In accord with the fed hyperinsulinaemia data, after the injection of insulin, the blood glucose levels of *GLUT4*-null mice remained significantly higher than in normal controls throughout the test period (Fig. 2*b*). In the fed state, *GLUT4*-null mice maintain nearly normal glycaemias but do show a significant increase in insulin concentrations (Table 1). This hyperinsulinaemia must work through mechanisms other than the stimulation of *GLUT4*

translocation to normalize the fed glycaemias in *GLUT4*-null mice.

Other serum metabolites were measured in *GLUT4*-null mice to determine the effect of *GLUT4* deficiency on glucose and fat metabolism. Fasted lactate levels in *GLUT4*-null mice were 30–58% lower and fed lactate levels were 47–70% lower than in normal mice (Table 1). These results suggest that the oxidation of glucose to lactate in skeletal muscles is depressed. Serum free fatty acid (FFA) levels in *GLUT4*-null mice were 10–20% lower in the fasted state and 23–45% lower in the fed state than in controls (Table 1). The lower serum FFA levels in fed mice are consistent with the postprandial hyperinsulinaemia in *GLUT4*-null mice. There is also an almost ninefold decrease in β-hydroxybutyrate concentration in the fasted serum of *GLUT4*-null mice compared with normal mice (Table 1). The lower FFA levels in the fasted serum of *GLUT4*-null mice could be due to increased FFA utilization in the muscles or to a decreased availability of FFAs. Most three-month-old male and female *GLUT4*-null mice have significantly diminished internal fat deposits (Fig. 2*a*). As illustrated in Fig. 2*a*, a 3.5-month-old

TABLE 2 Heart weight to body weight comparisons of *GLUT4*-null and wild-type mice

	Body weight (g)	Heart weight (g)	Heart/body
Females			
Control weight matched	18.5 ± 1.2 (4)	0.115 ± 0.01* (4)	0.006 ± 0.00042* (4)
Control age matched	28.2 ± 0.8* (4)	0.121 ± 0.004* (4)	0.004 ± 0.00018* (4)
<i>GLUT4</i> -null	21.04 ± 0.91 (8)	0.216 ± 0.011 (8)	0.01 ± 0.001 (8)
Males			
Control weight matched	27.4 ± 1.4 (5)	0.158 ± 0.005‡ (5)	0.006 ± 0.00024* (5)
Control age matched	40.7 ± 3.7† (6)	0.152 ± 0.013‡ (6)	0.004 ± 0.00044* (6)
<i>GLUT4</i> -null	27.5 ± 5.0 (3)	0.255 ± 0.035 (3)	0.009 ± 0.001 (3)

Body weights were taken before necropsy. Hearts were removed, rinsed free of blood with PBS, dried and weighed. Statistical analysis was done with a two-tailed unpaired Student's *t*-test. All values represented are means ± s.e.m. Significance was accepted at *P* ≤ 0.05. Numbers in parentheses are numbers of individuals.

* *P* ≤ 0.001; † *P* ≤ 0.01; ‡ *P* ≤ 0.05.

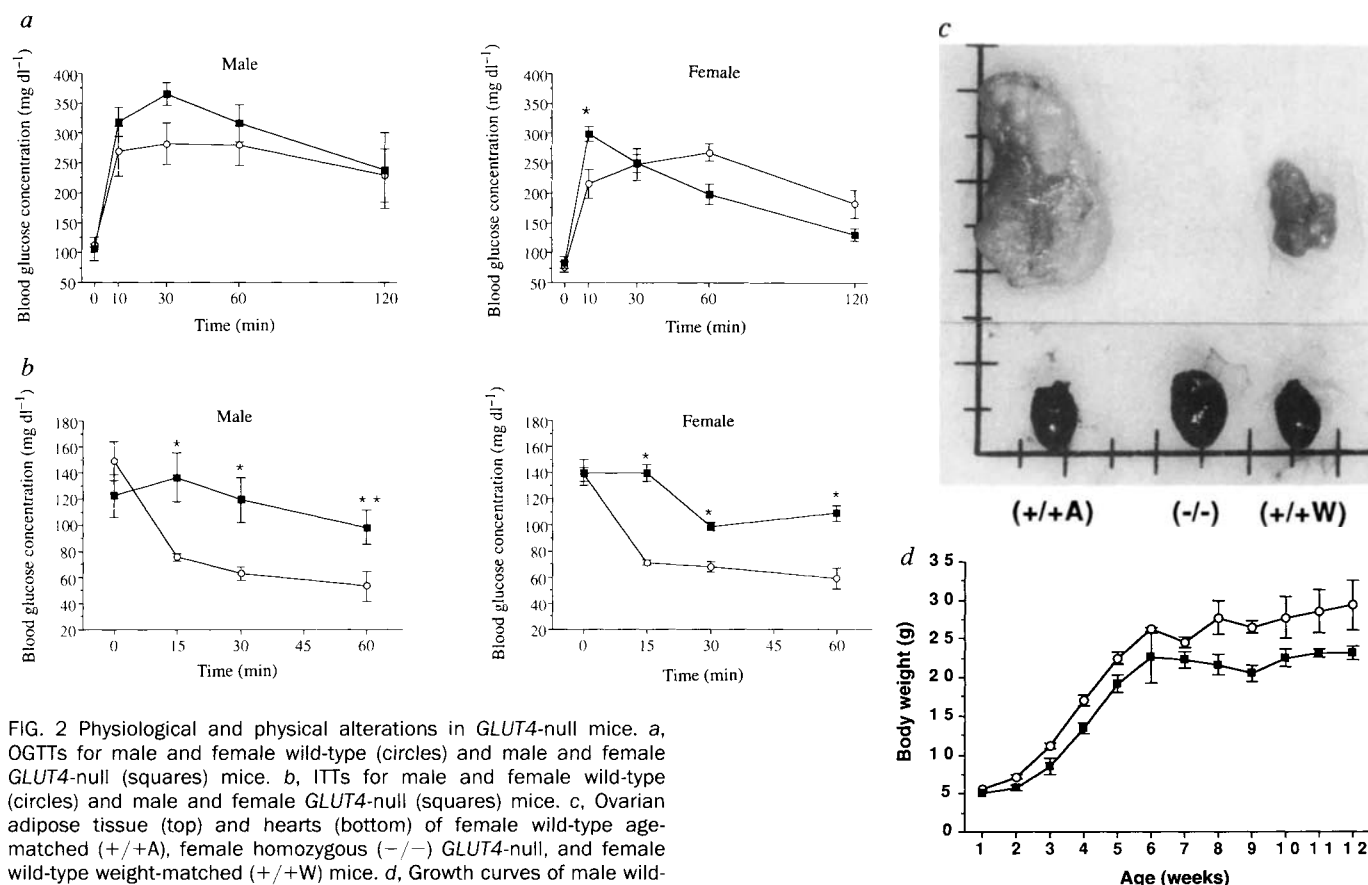


FIG. 2 Physiological and physical alterations in *GLUT4*-null mice. **a**, OGTTs for male and female wild-type (circles) and male and female *GLUT4*-null (squares) mice. **b**, ITTs for male and female wild-type (circles) and male and female *GLUT4*-null (squares) mice. **c**, Ovarian adipose tissue (top) and hearts (bottom) of female wild-type age-matched (+/+A), female homozygous (-/-) *GLUT4*-null, and female wild-type weight-matched (+/+W) mice. **d**, Growth curves of male wild-type (circles) and male *GLUT4*-null (squares) mice.

METHODS. **a**, After a 16-h fast, 4 wild-type males, 4 wild-type females, 5 *GLUT4*-null males and 4 *GLUT4*-null females, all 10 weeks of age, were given 0.5 mg glucose per gram body weight orally through a gavage needle. Blood was withdrawn from the orbital sinus at 0, 10, 30, 60 and 120 min. Blood glucose levels were measured with a One Touch II monitor (Lifescan) on whole blood, or by the glucose oxidase method (Sigma) on serum. Values are means $\pm$ s.e.m. Statistics were performed with repeated measure analysis (SuperANOVA, Abacus Concepts). Asterisk represents $P \leq 0.01$. **b**, After a 6-h fast, 5 wild-type males, 5 wild-type females, 5 *GLUT4*-null males and 5 *GLUT4*-null females, all 10 weeks of age, were given 0.75 U porcine insulin per kg body weight by intraperitoneal injection²³. Blood was withdrawn from the orbital

sinus at 0, 15, 30 and 60 min. Blood glucose levels were measured with a One Touch II monitor (Lifescan) on whole blood or by the glucose oxidase method (Sigma) on serum. Values are means $\pm$ s.e.m. Statistics were performed as in **a**. Significance levels: *, $P \leq 0.01$ and **, $P \leq 0.05$. **c**, Necropsies were performed on a 3.5-month-old wild-type female age-matched control (+/+A) (left); a 3.5-month-old *GLUT4*-null (-/-) (centre); and a 2-month-old weight-matched control (+/+W) (right). The adipose tissue (top panel) and the hearts (bottom panel) of female mice are shown. **d**, 12 male wild-type and 10 *GLUT4*-null mice were weighed once a week on the same day and time. Values are means $\pm$ s.e.m.. Statistics were performed as in **a**. Similar growth patterns were observed in female mice (results not shown).

female *GLUT4*-null mouse had no dissectable ovarian fat pad. The dramatic reduction in adipose tissue seen in *GLUT4*-null mice contrasts with the increased adiposity seen in mice that overexpress *GLUT4* in their fat¹². It has been proposed that non-insulin-dependent diabetes could be considered a disease caused by dysfunctions in lipid metabolism that lead ultimately to alterations in glucose metabolism¹³. The decreased lipids in the serum of *GLUT4*-null mice are the opposite of the serum lipid profile characteristic of the diabetic phenotype¹⁴. The diminished adipose deposits and the decreased availability of FFAs in *GLUT4*-null mice may be important in the maintenance of non-diabetic glycaemias.

Morphological abnormalities brought about by *GLUT4* deficiency are observed. *GLUT4*-null mice are growth-retarded (Fig. 2d). At necropsy, 2–4-month-old female and male *GLUT4*-null mice show significant heart hypertrophy (Fig. 2c). *GLUT4*-null mice have heart weight to body weight ratios 2.3 to 2.5 times those of age-matched controls and 1.4 to 1.5 times those of weight-matched controls (Table 2). This cardiac hypertrophy could be the result of the observed hyperinsulinaemia seen in *GLUT4*-null animals, which has been postulated to contribute to cardiac hypertrophy seen in other models^{14,15}. Another factor contributing to the observed cardiac hypertrophy could be the decreased supply of FFAs because studies have shown that

inhibiting cardiac fatty-acid oxidation can result in hypertrophy^{16,17}. Finally, *GLUT4*-null mice have decreased longevity, with most animals dying at 5–7 months, possibly owing to cardiac function abnormalities associated with the observed severe hypertrophy.

The results demonstrate that *GLUT4*-null mice can compensate for the lack of the insulin-sensitive glucose transporter and maintain near-normal levels of blood glucose. However, perturbations in cellular glucose and lipid metabolism, cardiac hypertrophy, severe reduction of adipose tissue, and growth retardation result from deleting the gene for *GLUT4*. An understanding of the compensatory responses to the lack of *GLUT4* is likely to reveal mechanisms that could be exploited in the treatment of diabetes. □

Received 3 March; accepted 20 July 1995.

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ACKNOWLEDGEMENTS. E.B.K. and A.E.S. contributed equally to this work, which is submitted in partial fulfilment of the requirements for the Ph.D. degree for the Albert Einstein College of Medicine (A.E.S.). We thank L. Huang for technical assistance; R. Burcelin, J. Li, V. Schramm, S. Seifter and T. S. Tsao for discussions and critical comments; R. Jaenisch for the gift of the 129/SV λ library; M. Rudnicki and R. Jaenisch for the gifts of the NEO and TK plasmids; and E. J. Robertson for the gift of the WW6 embryonic stem cells. This work was supported by grants from the Pew Charitable Trust, the Life and Health Insurance Medical Research Fund, the Diabetes Research and Education Foundation, and the Albert Einstein Cancer Center (M.J.C.). E.B.K. was supported by postdoctoral fellowships from the Martin Foundation and the New York Community Trust; A.E.S. was supported by predoctoral fellowships from the Medical Scientist Training Program and the NIH; K.H. was supported by the NIH; R.D. was supported by a James S. McDonnell Scholar Award; and M.J.C. is a recipient of a career development award from the American Diabetes Association.

Functional MRI evidence for adult motor cortex plasticity during motor skill learning

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PERFORMANCE of complex motor tasks, such as rapid sequences of finger movements, can be improved in terms of speed and accuracy over several weeks by daily practice sessions. This improvement does not generalize to a matched sequence of identical component movements, nor to the contralateral hand. Here we report a study of the neural changes underlying this learning using functional magnetic resonance imaging (fMRI) of local blood oxygenation level-dependent (BOLD)^{1–4} signals evoked in primary motor cortex (M1). Before training, a comparable extent of M1 was activated by both sequences. However, two ordering effects were observed: repeating a sequence within a brief time window initially resulted in a smaller area of activation (habituation), but later in a larger area of activation (enhancement), suggesting a switch in M1 processing mode within the first session (fast learning). By week 4 of training, concurrent with asymptotic performance, the extent of cortex activated by the practised sequence enlarged compared with the unpractised sequence, irrespective of order (slow learning). These changes persisted for several months. The results suggest a slowly evolving, long-term, experience-dependent reorganization of the adult M1, which may underlie the acquisition and retention of the motor skill.

In the motor task used, the fingers of the non-dominant hand were opposed to the thumb in two specified sequences, A and B (Fig. 1a). Subjects were instructed to tap each sequence, without looking at their hand, as accurately and rapidly as possible.

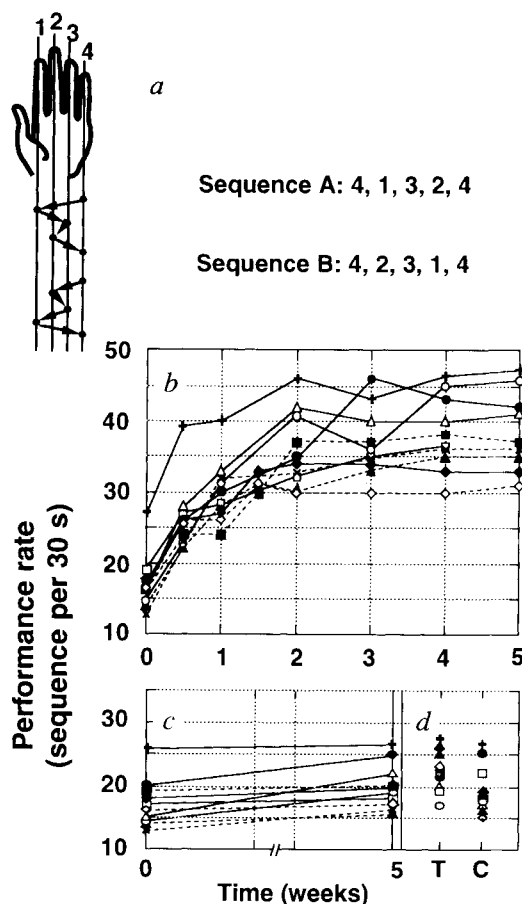


FIG. 1 a, The sequence of finger-to-thumb opposition movements. In Sequence A the order of finger movements was 4,1,3,2,4 (numbering the fingers from index to little), and in Sequence B the order was 4,2,3,1,4 as indicated by the arrows (matched, mirror-reversed sequences). Practice consisted of tapping the designated training sequence as fast and accurately as possible for 10–20 min per day, using the non-dominant hand only, light touch of finger pad to thumb pad, and without looking at the hand. Performance was measured in terms of speed and accuracy by two observers for a 30 s interval: one observer counted the number of sequences completed, the other the number of errors. b, Learning curves for the trained sequence. Each curve (symbol) depicts the performance of a single subject as a function of time. Pretraining (time point 0), day 3 and 10 of training, and performance on the day of the subsequent weekly imaging sessions is shown for 10 subjects: the 6 who underwent fMRI scanning, and 4 additional subjects (3 females, 1 male; 26–46 years old) who were tested in the behavioural task but were not scanned (broken lines). The number of complete sequences performed in a 30-s test interval (rate) increased from 17.4 ± 3.9 to 38.4 ± 5.8 (mean $\pm$ s.d.; week 0 and 5 weeks of training, respectively; paired t -test, $P < 0.001$). Accuracy also improved, with the number of sequences that contained errors decreasing from a mean of 2.4 ± 0.9 to 0.5 ± 0.5 (paired t -test, $P < 0.001$). (Although some subjects could perform the task at a rate of up to 7 component movements per second at the later stages of training, there was good inter-observer agreement in error counting (mean difference of 0.7 ± 0.7 , 2 observers, 10 measurements).) c, There was no significant improvement for the control sequence (performance rate 18.1 ± 3.7 to 19.4 ± 4.2 ; 0 and 5 weeks of training, respectively; paired t -test, n.s.). d, There was little or no transfer of the learning effect to the contralateral (dominant) hand. Trained (T) versus control (C) sequence performance rates, at week 5, were 22.3 ± 2.9 and 19.8 ± 4.0 , respectively (paired t -test, $P = 0.097$).

Initial performance was measured for both sequences immediately before the first scanning session. Thereafter, each subject was randomly assigned one sequence (trained) to be practised for 10–20 minutes each day for several weeks, whereas the other sequence (control) was to be performed only during scanning.

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Initial performance of the two sequences did not differ (Fig. 1b). However, practice had a large effect on performance: the speed at which the trained sequence could be performed increased across several consecutive sessions, reaching asymptote after approximately 3 weeks of training in all subjects, with more than doubling of the initial rate and a concurrent gain in accuracy. This improvement was specific to the trained hand, with little or no transfer of the learning effect to the contralateral hand (Fig. 1d). Moreover, learning did not generalize to the performance of the control sequence, although both sequences were made up of identical component movements (Fig. 1c). These results suggest that a lateralized, discrete representation of the learned sequence undergoes experience-dependent changes.

We investigated whether these changes in performance would be reflected in the evoked BOLD signal in M1. Six adult male subjects, 24–44 years old (five right-handed, one left-handed) were scanned once a week for 4–6 consecutive weeks in a 4 T MRI system equipped with echo planar imaging capabilities⁵. Each session consisted of 6–10 experimental sets. The alternation of experimental conditions over time, within a set, is shown in Fig. 2. The general model was rest, first activation (X1), rest, second activation (X2), rest, where X1 and X2 were, respectively: sequence A then B; B then A; B then B; or A then A. Throughout the study, during scanning, both the trained and the control sequence were performed at a fixed, slow rate of two

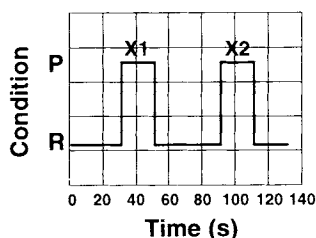


FIG. 2 The alternation of experimental conditions over time within an imaging set (R, rest; P, sequence performance). Each set was 128 s long: 28 s of rest, then 20 s of motor performance (X1), 40 s of rest, second interval of 20 s of motor performance (X2), and a final 20 s rest interval. Images were collected every 2 s.

METHODS. Data were processed by three separate routines, all software written in Interactive Data Language (Research Systems, Inc.). First, pixel-by-pixel Z-maps for each of the two motor performance intervals (X1 and X2) were constructed; this consists of dividing the difference between the interval of task performance and its immediately preceding and following rest periods by the noise value calculated from the first rest period²³. Second, a pixel-by-pixel percentage change map of signal intensity was calculated, again for each motor performance interval relative to the average of the rest intervals. As a final control, sample data sets were analysed using zero-mean, unit-variance, cross correlation of the detrended intensity changes for each pixel and a square-wave function corresponding to the alternation of conditions. As BOLD responses lag relative to the initiation and termination of movements^{1,24} (Fig. 4b, c), 3 scans were discarded in each transition between conditions in the set. Comparisons were then made, always within the set, and for each slice, between the number of pixels exceeding threshold in M1 during intervals X1 and X2, at arbitrary thresholds of $Z > 2.0$, $\Delta\% > 2.0$ and $r > 0.7$. A balanced randomized design was used. Pairs of sets, one made of X1 being A and X2 being B, and the other with A and B in reversed order, were run. The order of these sets within a pair was randomized. Control sets in which a given sequence was repeated twice within the set (at intervals X1 and X2) were run at the beginning and end of each session. Performance during scanning sessions was monitored for accuracy and rate by two observers. A set was repeated during the same session if more than a single error in the performance of the sequence was noted: this occurred only once. Having all comparisons made within sets avoids problems of head motion and drifts in absolute signal intensities between sets and between sessions. Sets showing evidence of within-set head motion were discarded and replaced by data sets acquired within one week. All 3 types of analysis yielded statistically significant effects. Only the Z-score map results are shown here.

component movements per second, paced by the loud magnetic field gradient switch noise. Thus both rate and component movements were matched, and the only difference between sequences during imaging sessions was the difference in practice histories. Data analysis consisted of determining those pixels in which signal intensity changed, relative to the level at rest, during interval X1 and X2 in each set, and then comparing the extent of the two statistical maps generated from each set.

In session 1, before training, a comparable extent of M1 was activated by the execution of both A and B (41.8 ± 12.2 and 42.3 ± 10.8 ; number of pixels summed over slices and averaged over sets for six subjects, respectively; mean $\pm$ s.d.). However, in the early sets of session 1 a strong ordering effect was found. Either sequence consistently resulted in a larger area of evoked response when executed first rather than second within the set, that is, the area of the evoked BOLD signal, irrespective of sequence type, was greater for X1 than X2. By the latter part

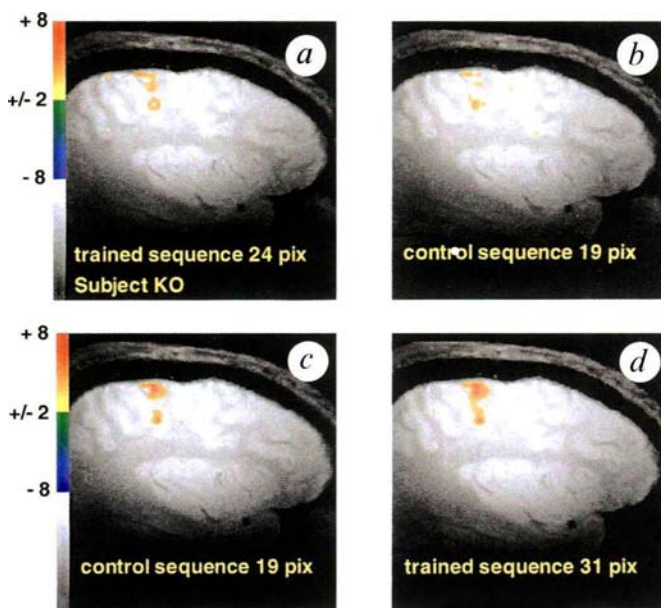


FIG. 3 Differential evoked responses to the trained versus control sequence following 3 weeks of daily practice by subject KO on the designated training sequence, using his left hand; session 4. A parasagittal plane of section through the right hemisphere centred 35 mm from midline is shown; right, anterior; top, dorsal aspect of the brain. Activation maps from two sets (top and bottom) are shown superimposed on the corresponding structural MR image. Top, trained sequence performed first in the set (a) versus the untrained sequence performed second (b). Bottom, untrained sequence performed first (c) versus the trained sequence performed second (d). Z-score values are indicated by the pseudo-colour scale. The area of evoked response in M1 for the trained sequence was larger in extent irrespective of the order in which the sequences were performed. It did not, however, extend beyond the hand representation itself, as indicated by control experiments conducted in the initial and fifth session in 2 subjects, in which we mapped the evoked signal for each digit's flexion–extension movements, all four digits-to-thumb opposition, wrist extension–flexion, and eye (orbicularis oculi) closure (results not shown).

METHODS. Images were collected by a 4 T MRI system (Oxford magnet, Omega console), equipped with echo planar imaging (EPI) capabilities and a radio frequency (RF) surface coil. Sequence parameters: gradient-echo EPI, repetition time (TR) = 2 s, echo time (TE) = 26 ms, field of view (FOV) = 160 mm, resolution = 64 by 64. Four contiguous parasagittal slices centred about 35 mm from midline with slice thickness of 5 mm each were acquired, ensuring that all of the contralateral hand representation area was scanned². Subjects' heads were immobilized using foam pads. The EPI images were superimposed (using a two-dimensional warping algorithm) on structural MR images collected at the end of each session in the same plane of section. These were used to identify the central sulcus. Analysis was restricted to M1–anterior bank of the central sulcus and the precentral gyrus²⁵.

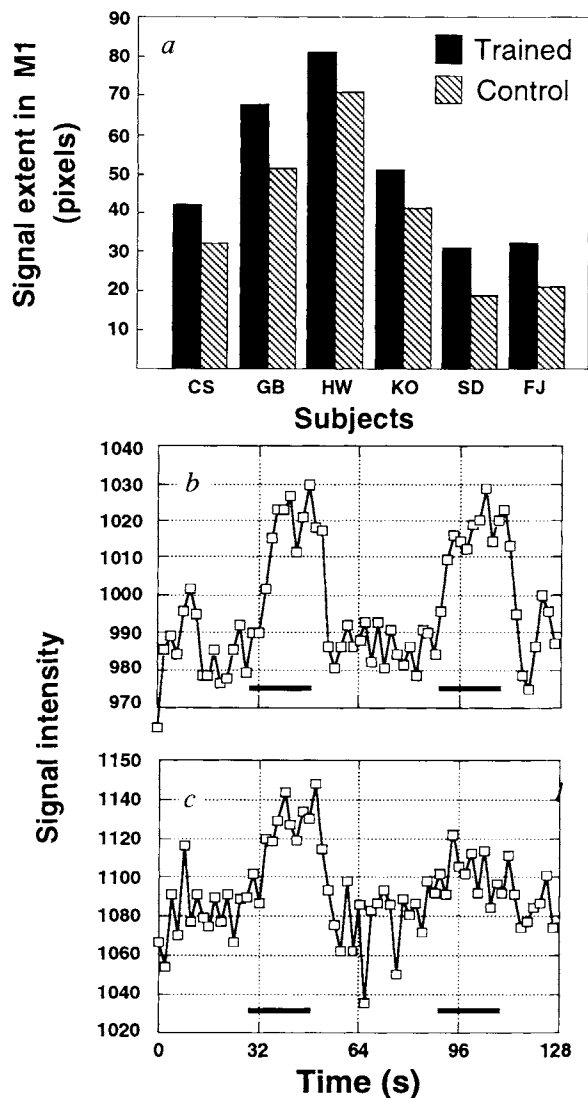


FIG. 4 *a*, The extent of M1 cortex responding for the trained versus untrained sequences in session 4, for 6 subjects (48.7 ± 21.9 and 39.7 ± 19.4 pixels, respectively; mean, s.d.; paired *t*-test, $P < 0.001$). For each subject the number of pixels was summed over slices and averaged over sets in the session to yield the total number of pixels responding for each sequence throughout the volume studied. *b*, Time-course data from a single pixel (2.5×2.5 mm) in M1, recorded during session 4, showing signal intensity changes within a set in which the subject executed the trained sequence during interval X1 and the control sequence during X2. Horizontal bars, intervals of motor performance. The activity-dependent change in intensity was of a similar magnitude for both sequences. *c*, Time-course data from a different pixel in M1, taken from the same set as above. The trained, but not the control, sequence evoked a significant intensity change. No significant differences were found in the mean signal intensity levels, relative to baseline, evoked in M1 by the performance of the trained versus the untrained sequence.

of session 1, however, by which time each subject had typically performed six two-minute experimental sets over the course of approximately 30 minutes, the ordering effect was reversed. A larger extent of M1 was activated by a given sequence when executed second rather than first in the set (greater for X2 than X1). This reversal of the ordering effect was specific to the sequences that were repeatedly performed during the session; when a new, third, motor sequence was introduced, again consisting of the same component movements (4,3,1,2,4; Fig. 1*a*), the initial habituation-like response^{6,7} to repetition returned. This specificity suggests that the switch in ordering effects may

reflect learning on a short time scale (fast learning). The conjecture is supported by the finding that the paced, repetitive execution of a sequence, as during the initial scanning session, resulted in a significant improvement in both speed and accuracy (correct sequences per 30 s increasing from 17.8 ± 3.3 to 22.5 ± 4.0 ; paired *t*-test, $P < 0.001$; number of errors decreasing from 2.6 ± 0.8 to 1.6 ± 0.5 ; paired *t*-test, $P < 0.05$; 6 subjects).

The reversed ordering effect was, however, gradually overridden by the effects of continued training (slow learning). By session 4, which corresponds to three weeks of daily practice on the designated training sequence, and for all subsequent sessions, the activation map evoked by the trained sequence was consistently larger in extent than the map evoked by the control sequence, irrespective of the order in which the sequences were executed (Fig. 3). The emergence of this consistent difference in the evoked MRI signal corresponded in time to the attainment of maximal asymptotic performance on the trained sequence. Figure 4*a* depicts this differential response for all six subjects after three weeks of daily practice.

Does the differential signal in M1 reflect an increase in signal amplitude or an expansion in the cortical representation? In analysing pixels that showed a response to both the trained and control sequences, we found that the activity-dependent change in signal intensity was similar for both sequences (Fig. 4*b*). However, there was a subpopulation of pixels that exceeded threshold in the trained sequence map but showed little or no response during the execution of the control sequence (Fig. 4*c*). This result indicates that, with practice, the extent of the trained sequence representation in M1 does indeed expand. We conjecture that motor practice induces the recruitment of additional M1 units into a network specifically representing the trained motor sequence. This interpretation is in agreement with findings in the monkey of cortical changes associated with motor, as well as perceptual skill, learning^{8,10}. One substrate for the enlargement of cortical representations may be the unmasking of pre-existing lateral connections between populations of neurons whose outputs result in different sets of movements¹¹. This can occur on a short time scale, and may subserve fast learning. However, to account for the gradual evolution of asymptotic performance as well as the differential cortical responses, we suggest that improved synaptic connections between task-relevant units subserving the execution of the acquired skill are formed as the result of long-term practice^{12,13}. Indeed, results from two subjects have shown the persistence of the differential evoked signal in M1, as well as superior performance, over intervals of 10 and 21 weeks of no additional training (results not shown).

This study was designed to examine local cortical changes in the adult motor cortex that result from long-term practice. Several positron emission tomography (PET) studies have examined short-term changes occurring as a consequence of practice in motor^{14,17} and sensorimotor^{18,19} tasks within a single session. Although some studies have suggested that, as learning proceeded within the session, blood flow in M1^{14,17,19} (and the supplementary motor area^{16,18}) increased, no significant M1 changes have been found when the rate of movements in the trained and untrained conditions were controlled^{15,16}. A consistent decrease in activation in the cerebellum^{14,16} and prefrontal¹⁶ cortex (with conflicting observations concerning premotor cortex^{16,17,19}) was, however, found to occur with practice within a session even independently of performance rate. Because the decrease in activation in areas projecting to M1 occurred over a similar time scale as the switch in ordering effects on repetition that we observed in M1 within the first session, we suggest that the switch reflects changes in inputs to M1. Moreover, the switch in processing mode, dependent on a critical amount of practice, may constitute an important step in initiating ('gating') subsequent experience-dependent changes in M1. This interpretation may resolve some apparently contradictory transcranial magnetic stimulation studies^{20,21}; although sequence performance induced

a decreasing area from which motor responses could be evoked, once a sequence of movements was made explicit²¹, the area from which a response could be evoked expanded across five consecutive daily sessions²⁰. Our results suggest that motor skill learning has two distinct phases, analogous to those subserving perceptual skill learning¹². First, a within-first-session switch in processing mode is induced, which may be thought of as the acquisition of a task-relevant routine or set (perhaps an indication that a specific sequence of movements constitutes a special entity of behavioural significance). Second, there follows a much slower between-session phase of learning, which may reflect the gradual evolution of a specific representation of the skilled movements requiring long-term practice over several weeks to be completed^{10,12,22}. The final outcome of training is a new, more extensive representation of the trained sequence in primary motor cortex, which may constitute a site for the long-term memory of the skill in the adult human brain. □

Received 3 February; accepted 27 July 1995.

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ACKNOWLEDGEMENTS. We thank R. Desimone, P. DeWeerd, M. Hallett, B. Jagadeesh, M. Mishkin and D. Sagl for comments and discussions. A. Karni was supported in part by a J. William Fulbright Fellowship. R. Turner was supported by the Wellcome Trust and the Human Frontier Science Program Organization.

Changes in retinal cell fate induced by overexpression of EGF receptor

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THE differentiation of multipotential progenitor cells in the vertebrate retina into photoreceptors, neurons and glial cells is regulated in part by cell–cell signalling^{1–11}. Transforming growth factor (TGF)- α is one of the extracellular signals implicated in the control of several aspects of retinal development, including proliferation and cell fate^{5,6,11–13}. The way cells interpret pleiotropic signals such as TGF- α is influenced by the level of expression of epidermal growth factor receptor (EGF-R) in some cell lines^{14,15}. To address the influence of receptor level on responses of retinal progenitor cells to TGF- α , additional copies of EGF-Rs were introduced *in vitro* and *in vivo* with a retrovirus. Normally *in vitro*, low concentrations of TGF- α stimulated proliferation whereas high concentrations biased choice of cell fate, inhibiting differentiation into rod photoreceptors while promoting differentiation into Müller glial cells. We report here that introduction of extra EGF-Rs into progenitor cells *in vitro* reduced the concentration of TGF- α required for changes in rod and Müller cell differentiation but did not enhance proliferation. Introduction of extra EGF-Rs *in vivo* increased the proportion of clones that contained Müller glial cells, suggesting that receptor level is normally limiting. These findings demonstrate that responsiveness to extracellular signals during development can be modulated by the introduction of additional receptors, and suggest that the level of expression of receptors for these signals contributes to the regulation of cell fate.

Recent observations *in vitro* suggest that differences in both the availability of extracellular signals^{7–11} and the responsiveness of progenitor cells^{6,8,16} contribute to the sequential development of 7 different types of cells in the vertebrate retina. Although some of the differences among progenitor cells may reflect the presence or absence of appropriate receptors for extracellular signals, recent studies with several cell lines suggest that the level of receptor expression may also play an important role in determining how cells interpret extracellular signals. For

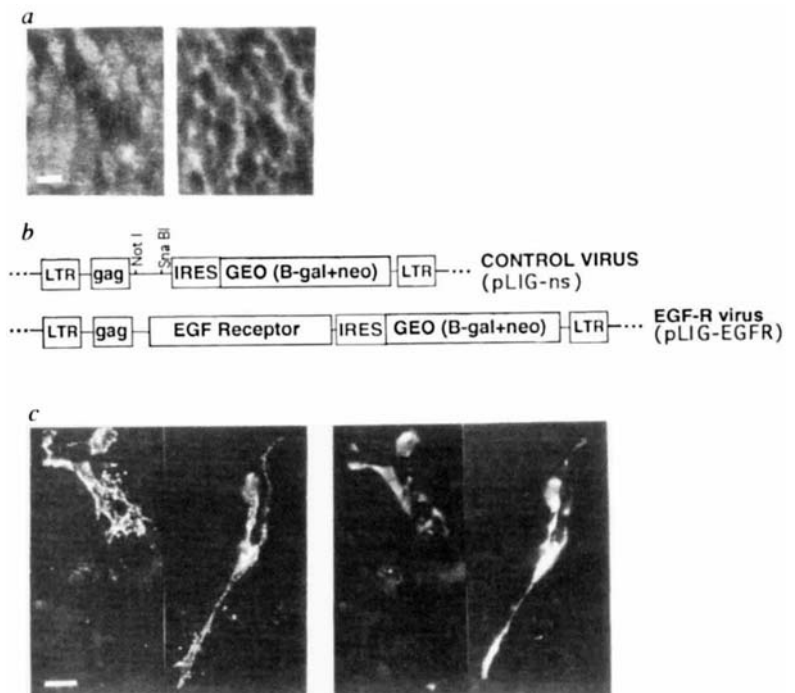
example, the introduction of additional copies of wild-type forms of EGF receptors was reported to increase 3T3 cell proliferation and frequency of transformation in response to EGF¹⁵, and change the response of PC12 cells to EGF from proliferation to differentiation¹⁴. To determine whether limitations in the level of EGF receptor expression influence the way retinal progenitor cells respond to TGF- α or related ligands, additional copies of wild-type EGF-Rs were introduced into progenitor cells in newborn rat retinas *in vitro* and *in vivo*. At this age, retinal progenitor cells express endogenous EGF receptors (Fig. 1a), and several ligands that activate EGF-Rs, including TGF- α , are expressed in the developing retina^{5,6,17}. To follow the fate of progenitor cells, a replication-incompetent retrovirus vector expressing a histochemical marker (B-geo; 'control virus', Fig. 1b) was used to label these cells. To assess the effects of extra EGF receptors on progenitor cell proliferation and differentiation, a virus that coexpresses the marker and wild-type human EGF receptors was produced ('EGF-R virus', Fig. 1b, c). The level of expression of EGF-Rs by cells infected with this virus was at least double the level of endogenous EGF-R expressed by neighbouring uninfected cells 4 days after infection (Fig. 2a).

Initial studies were performed *in vitro* with explants of newborn retina. Progenitor cells in explants divide and differentiate in parallel with progenitor cells *in vivo*¹⁸, providing a physiological system in which the concentration of ligand could also be modulated. The fate of progenitor cells was followed by infecting them when explants were prepared and analysing their proliferation and differentiation over a 2–3 week period. At this stage *in vivo*, multipotential progenitor cells generate rod photoreceptors, bipolar and amacrine neurons, and Müller glial cells¹.

Progenitor cells infected with control virus in explants developed predominantly into rod photoreceptors, the normal fate of most progenitor cells in newborn rat retina *in vivo* (Fig. 3a)¹. Exogenous TGF- α (1–100 ng ml⁻¹) antagonized development into rods (Fig. 3a)¹¹. In some cells, rod development appeared to be delayed rather than inhibited: the proportion of cells expressing rod markers rose from 26% of control (untreated) levels to 54% between 2 and 3 weeks *in vitro* in 10 ng ml⁻¹ TGF- α . In other cells, TGF- α inhibited development into rods, promoting development into Müller glial cells instead (Fig. 3b–d). Müller cells were identified by their larger size and expression of vimentin, glutamine synthetase (GS), and glial fibrillary acidic protein (GFAP)^{19,20}. During the first week *in vitro*, TGF- α stimulated proliferation in explants (Fig. 3e), as previously reported⁶; however, optimal effects on proliferation were

FIG. 1 Retinal progenitor cells express endogenous EGF-Rs, and coexpress virally transduced human EGF-Rs and the histochemical marker *geo*(β -gal + neo). **a**, Many retinal progenitor cells, identified by their expression of PCNA cyclin (left panel), also express EGF-Rs (right panel), in a section of newborn rat retina. **b**, Constructs used to generate virus expressing a histochemical marker β -geo (control virus) or coexpressing the wild-type human EGF receptor and the marker β -geo (EGF-R virus). The constructs contain a single promoter in the Moloney murine leukaemia virus LTR and an ATG⁻ fragment of *gag* from pBabe-puro²⁵, β -geo (β -galactosidase fused to neomycinophosphotransferase)²⁶, and the IRES sequence from encephalomyocarditis virus (EMCV)²⁷. The IRES sequence allows translation of β -geo to be initiated from an internal site, using an ATG in the IRES sequence, and facilitates high levels of coexpression of two genes²⁸. **c**, Four retinal cells infected with EGF-R virus at PO coexpress the virally transduced human EGF receptor (left panel) and β -geo (right panel), stained 5 days after infection. Of the retinal cells infected with EGF-R virus that expressed β -geo, 95–100% also expressed the receptor, and 80–90% of infected cells that expressed the receptor also expressed β -geo when stained with anti- β -galactosidase antiserum and an antibody specific for the human, virally transduced EGF receptor 5–14 days after infection. Expression of β -galactosidase can therefore be used to monitor expression of the receptor. Scale bar, 5 μ m in **a**, 20 μ m in **c**.

METHODS. Newborn retinas were fixed overnight at 4 °C in 10% formalin, and frozen sections (10 μ m) were double labelled with anti-PCNA cyclin (mouse, Sigma) and anti-EGF-R (sheep, UBI) antibodies, visualized with donkey anti-mouse Texas red and goat anti-sheep fluorescein, respectively (Jackson ImmunoResearch). The specificity of the EGF-R antibody was confirmed by western blot analysis of PO retinal lysates (not shown). To generate virus coexpressing the human EGF-R and β -geo, the 4.2-kb *SacII*–*XhoI* cDNA fragment from pC011-EGFR-neo¹⁵, which includes the complete coding sequence for the wild-type human EGF receptor, was subcloned into pBlueScript (Stratgene) after changing the *SacII* site to a *XhoI* site. After digestion with *NotI* and *SacI*, the 3.9 kb fragment was inserted into the *NotI*–*SnaBI* sites of pLIG-ns (8.4 kb) to generate pLIG-EGFR (12.3 kb). pLIG-ns contains a 581 nucleotide IRES sequence from EMCV²⁷, directing translation of β -geo²⁶. Ψ 2 cells, an ecotropic packaging line²⁹, were transfected with 1 μ g plasmid DNA (pLIG-ns or pLIG-EGFR) using Lipofectamine (BRL). Clones of G418-resistant Ψ 2 cells that generated virus with titres of $4\text{--}6 \times 10^6$ CFU ml⁻¹ (unconcentrated) were selected, and virus was collected and stored as described previously²⁹. Expression of the appropriate 175K EGF-R protein¹⁵ was observed on



western blots of lysates from 3T3 cells infected with EGF-R virus, using an antibody specific for the human receptor (clone LA1, UBI) (not shown). Coexpression of the human EGF receptor and β -geo by infected retinal cells was confirmed by infecting explants of newborn or E18 retina with unconcentrated virus; after 5–14 days, explants were dissociated, replated on coverslips as described in Fig. 3 to help counting, and retinal cells were stained with anti-human EGF receptor antibodies (antibody 1 from Oncogene Science or clone LA1 from UBI), which are specific for the virally transduced human receptor and do not label endogenous EGF receptors, followed by donkey anti-mouse Texas red (Jackson ImmunoResearch). β -geo expression was visualized with anti- β -galactosidase antiserum (rabbit, 5-Prime 3-Prime) followed by goat anti-rabbit FITC. Biological activity was confirmed using 3T3 and PC12 cells: after infection with EGF-R virus, proliferation of 3T3 cells and neurite formation by PC12 cells were enhanced in the presence, but not the absence, of EGF or TGF- α (not shown), as reported^{15,14}.

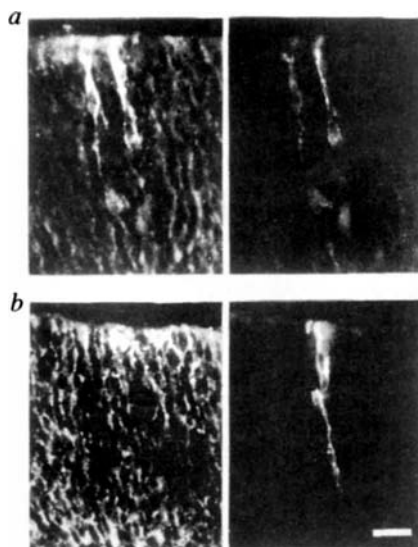


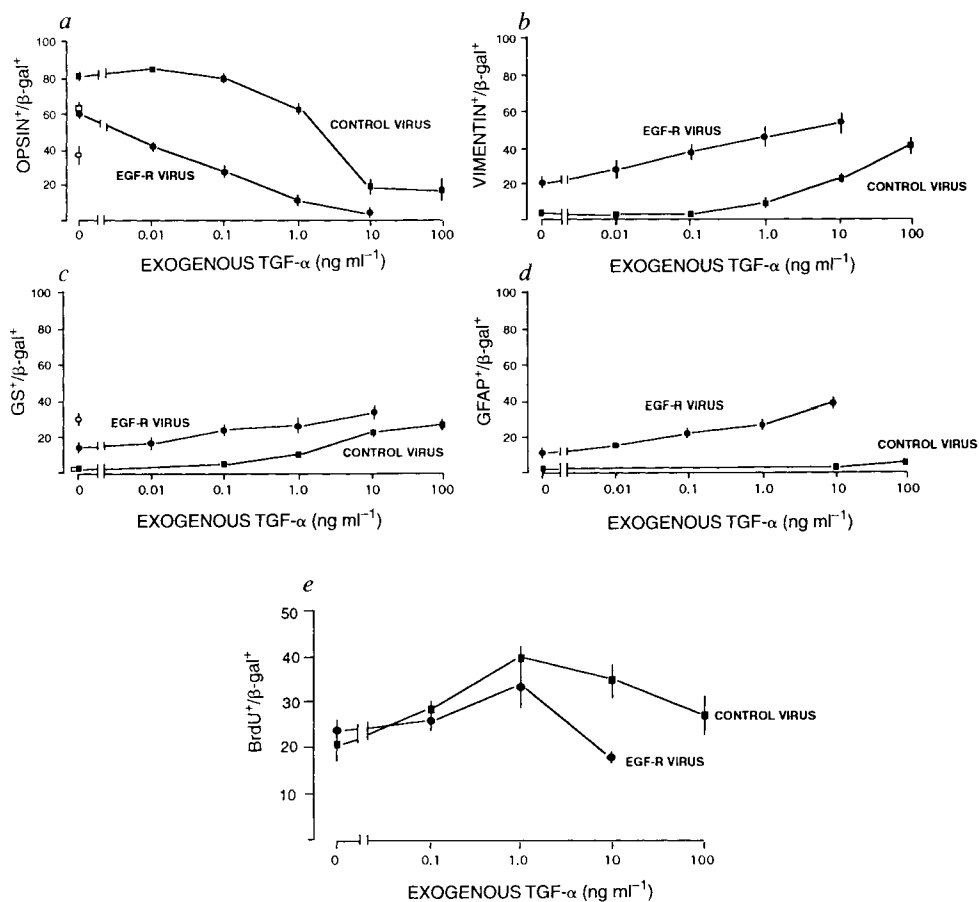
FIG. 2 Retinal progenitor cells infected at birth with EGF-R virus express higher levels of EGF-R (**a**) than uninfected neighbouring cells, while cells infected with control virus (**b**) express levels of receptor comparable to neighbouring uninfected cells. Expression of EGF-R was assessed by staining sections 4 days after infection with an antiserum that labels both endogenous EGF-receptors and the virally transduced human EGF-R (left panels). Infected cells were identified by double labelling with anti- β -galactosidase (right panels). The average EGF-R fluorescence intensity of cells infected with EGF-R virus was 2.4-fold higher (range of 1.5–3.9-fold) than that of uninfected neighbouring cells expressing endogenous EGF-Rs. The EGF-R fluorescence intensity of neighbouring uninfected cells varied over a 1.4–3.8-fold range (average: 2.8-fold). The highest levels of EGF-R fluorescence among cells infected with EGF-R virus were 1.6-fold (average) higher than the highest levels of fluorescence among neighbouring uninfected cells. Scale bar, 20 μ m. **METHODS.** Explants of newborn retina were infected as described in Fig. 3, fixed in 10% formalin 4 days later, and 10–20- μ m frozen sections were stained with sheep anti-EGF-R (UBI) and rabbit anti- β -galactosidase (5-Prime 3-Prime), visualized with donkey anti-sheep Cy3 and goat anti-rabbit fluorescein, respectively (Jackson ImmunoResearch). This EGF-R antibody recognizes both the endogenous rat EGF-R and the virally transduced EGF-R. Double-labelled sections were imaged on a BioRad MRC 600 confocal scanning microscope. To quantify differences in fluorescence intensity of cells stained with anti-EGF-R antiserum, peak intensity values of single line scans were determined from captured images. Twenty cells infected with EGF-R virus (identified by double-labelling with anti- β -galactosidase) and ~50 uninfected cells in the same fields were analysed.

FIG. 3 TGF- α and introduction of extra EGF-Rs affect progenitor cell differentiation and proliferation in explants infected at P0 with control virus (filled squares) or EGF-R virus (filled circles). a, TGF- α reduced the development of progenitor cells infected with control virus into rod photoreceptors after 2 weeks in culture. In progenitor cells infected with EGF-R virus, the concentration of TGF- α required for inhibition of rod development was reduced. Infected cells were identified by staining with anti- β gal antiserum, rods by expression of opsin³⁰. b, c, d, TGF- α increased the proportion of control virus-infected progenitor cells that developed into Müller glial cells, identified by expression of vimentin (b), glutamine synthetase (GS) (c) and glial fibrillary acidic protein (GFAP) (d) after 2 weeks in culture. The dose dependence of this effect was also shifted in EGF-R-infected cells. In addition to the expression of these antigenic markers, other features characteristic of Müller cells were also observed, such as increased size and astrocyte-like morphology in monolayer culture (not shown). Some of the cells that expressed vimentin did not appear to express GS or GFAP; these cells were not mitotic, and did not express markers characteristic of other types of retinal cells (not shown). They may represent either immature Müller cells or progenitor cells arrested in a post-mitotic, undifferentiated state. e, TGF- α enhanced proliferation of control virus-infected cells after 4 days in culture at low concentrations, although proliferation declined at higher concentrations that stimulated maximal effects on differentiation. In EGF-R virus-infected cells, no significant increase in proliferation relative to control virus-infected cells was observed, although like control virus-infected cells, proliferation was reduced at higher concentrations of TGF- α that supported maximal effects on differentiation (a–d). In a and c, results obtained from cultures grown in serum-free medium (see below) are shown as open squares for control virus-infected cells and open circles for EGF-R virus-infected cells.

METHODS. Explants of P0 retina (newborn, Sprague–Dawley rat) were cultured on nucleopore filters (13 mm diameter, 0.2 μ m pore; Costar) floating in 1.5 ml of culture medium in 35 mm tissue culture dishes^{6,8}. Explants (~0.75–1.0 mm²) were cut from the peripheral regions of retinas as described previously⁹ to minimize the number of astrocytes and cells associated with the vasculature. Culture medium consisted of DMEM-F-12 (1:1, Gibco-BRL) plus 5% fetal bovine serum (Gibco-BRL), 50 U ml⁻¹ penicillin, 50 μ g ml⁻¹ streptomycin (Gibco-BRL), and 5 μ g ml⁻¹ bovine insulin (Sigma). In some experiments, explants were cultured in serum-free medium containing N-2 supplements⁶. Similar effects were observed in the presence and absence of serum; however, overall survival was better in medium that contained serum. Explants

observed at lower concentrations than effects on differentiation. Moreover, proliferation was reduced at concentrations of TGF- α that elicited maximal effects on differentiation (Fig. 3). These observations suggest that increased Müller cell numbers were not likely to be due to selective proliferation.

The fate of progenitor cells in explants infected with EGF-R virus was altered even in the absence of exogenous TGF- α : rod development was reduced and Müller development was enhanced, presumably due to endogenous ligand(s) (Fig. 3). The endogenous ligand(s) may be TGF- α , or one of the other members of this family expressed in the retina^{5,6,17}. The concentration of exogenous TGF- α required for maximal effects on differentiation was also reduced in cells expressing extra EGF-Rs, by at least tenfold (Fig. 3). Similar effects were observed with human and rat TGF- α , and with EGF (not shown). No further increase in Müller cell differentiation was observed after an additional



were infected after placement on filters by adding a drop of virus-containing medium (15–20 μ l) to the tops of filters. This resulted in the infection of several hundred progenitor cells per explant. One day later, TGF- α (human recombinant, Collaborative Biomedical Products) was added to the 1.5 ml of medium in the dishes at the concentrations indicated, and 30 μ l of diluted TGF- α was added to the tops of filters. In some experiments, recombinant rat TGF- α (Sigma) or recombinant human EGF (R&D) were used instead of human TGF- α . Fresh TGF- α or EGF was added daily, and 0.5 ml of medium was replaced every 4 days. For analysis of proliferation, bromodeoxyuridine (BrdU; 2 μ M; Boehringer) was added after 3 days *in vitro*. To stain cells after 4 or 14 days in culture, explants were dissociated with trypsin (0.1%; Sigma) for 30 min, replated on poly-D-lysine-coated HTC slides (Cel-Line Associates) for 30–60 min, and fixed with 4% paraformaldehyde and 70% ethanol. To identify infected cells, slides were stained with anti- β -galactosidase antiserum (rabbit; 5-Prime 3-Prime), followed by goat-anti-rabbit FITC (Jackson ImmunoResearch); slides were then stained with antibody against opsin (RetP1; ref. 29), vimentin (Sigma), glutamine synthetase (Chemicon), GFAP (Sigma), or BrdU, followed by donkey anti-mouse Texas red (Jackson ImmunoResearch). Each point represents data from 3–9 cultures (mean $\pm$ s.e.m.) that contained 5 explants each.

week *in vitro*, though the proportion of infected cells that expressed the rod marker opsin rose (for example, 27% at 2 weeks versus 48% at 3 weeks in 0.1 ng ml⁻¹ TGF- α). Why is differentiation choice biased in some cases and only delayed in others? Preliminary observations indicate that choice is more likely to be biased if infections are initiated earlier (L.L., manuscript in preparation), suggesting that bias versus delay could reflect the degree of the inclination towards rod development. More progenitor cells in older retinas may already be inclined towards rod development, resulting in delayed differentiation instead of biased choice of cell fate.

Several observations suggest that the over-representation of Müller cells following overexpression of EGF-Rs was not due to their selective survival or proliferation. First, introducing extra EGF-Rs inhibited proliferation at concentrations of TGF- α that elicited maximal effects on Müller cell development (Fig. 3).

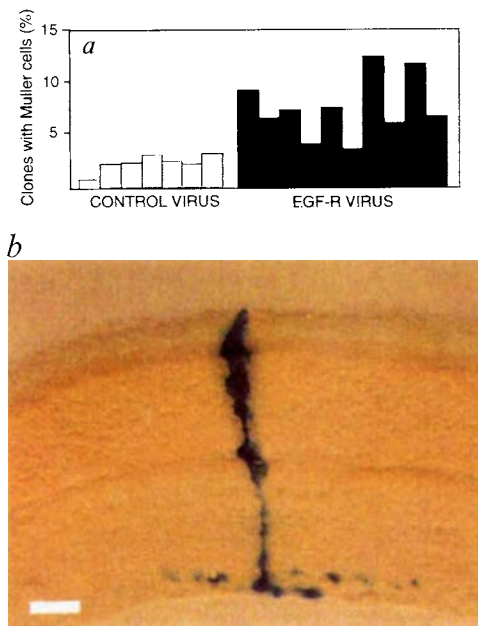


FIG. 4 Effects of EGF-R virus on Müller cell development *in vivo*. **a**, Comparison of the proportion of clones containing Müller cells in three week-old retinas infected at birth with control virus or EGF-R virus. Each bar represents an individual retina. A total of 4,161 clones in 7 control virus-infected retinas and 2,355 clones in 10 EGF-R virus-infected retinas were counted. **b**, A representative clone of EGF-R virus-infected cells, containing 1 Müller cell and 5 rods. Müller cells were identified by their unique morphological features: a cell body located in the inner nuclear layer and processes that extend to the inner and outer limiting membranes. Scale bar, 20 μ m.

METHODS. The retinas of newborn rats were injected as described¹, using $\sim 1 \mu$ l unconcentrated virus per retina (inoculum also contained 0.025% fast green, 80 μ g ml⁻¹ polybrene, and 10% fetal bovine serum). After 3 weeks, retinas were dissected, processed for X-gal histochemistry as whole mounts^{1,29}, embedded in gelatin (7.5%), frozen and sectioned (30–50 μ m). Cell type was defined by laminar position and morphological features¹.

Second, in explants of E18 retina, which contain few if any Müller cells, just 2 days of exposure to TGF- α (1–10 ng ml⁻¹) resulted in the expression of morphological features of Müller cells by 50–60% of cells infected with EGF-R virus, arguing against selective survival. Third, delaying exposure to TGF- α for 1 week in explants of newborn retina did not result in a reduction in rods (68% opsin⁺, compared to 64% in untreated cultures), or an increase in Müller cells (11% GS⁺ compared to 13% in untreated cultures), indicating that elevated activation of EGF receptors does not cause the selective death of rods or stimulate the proliferation of Müller cells. Additional support for this interpretation comes from clonal analysis *in vivo* (see below). The effects of manipulating the levels of TGF- α and EGF-Rs *in vitro* therefore indicate that enhanced sensitivity to ligand can be achieved by the introduction of extra EGF receptors, and suggest that limitations in receptor expression as well as ligand availability contribute to the regulation of retinal cell fate.

To determine whether limitations in receptor expression normally contribute to the regulation of cell fate *in vivo*, retinas of newborn rats were infected with either control or EGF-R virus, and clone size and cellular composition were analysed 3 weeks later. In retinas infected with control virus, 1–3% (average: $2.4 \pm 0.3\%$) of the resulting clones contained Müller cells (Fig. 4a), as previously reported¹. By contrast, in retinas infected with EGF-R virus, 3.5–12% (average: $7.6 \pm 1.0\%$) of the clones contained Müller cells (Fig. 4a). Most of these clones contained a single Müller cell in combination with 1–6 other

cells, usually rods (Fig. 4b), as observed previously *in vivo*¹. Average clone size was not significantly different among EGF-R and control virus-infected clones (1.8 ± 0.06 cells per clone for control virus versus 1.9 ± 0.05 cells per clone for EGF-R virus), consistent with observations in explants (Fig. 3e). These findings indicate that sufficient endogenous ligand is present *in vivo* to drive the activation of the extra, virally transduced receptors, suggesting that normally the level of expression of endogenous receptors is limiting. As observed *in vitro*, increased activation of EGF receptors *in vivo* increased the probability of generating Müller glial cells.

Observations *in vitro* and *in vivo* suggest that different thresholds of EGF receptor activation may underlie different responses. For example, low concentrations of exogenous TGF- α stimulated proliferation in normal explants, whereas high concentrations biased choice of cell type. The addition of extra receptors did not enhance the effectiveness of low concentrations of ligand for mitotic responses, but did enhance the effectiveness of a given concentration of ligand in biasing cell fate both *in vitro* and *in vivo*. These observations suggest that low levels of receptor activation may generate a mitotic response whereas higher levels affect cell type choice. This kind of correlation of proliferation versus differentiation responses with low versus high levels of EGF-R expression and activation was recently reported for PC12 cells¹⁴. Preliminary observations indicated that the normal level of EGF-R expression in the retina increases during late embryonic and early postnatal development; at times when endogenous receptor expression is low, introduction of extra receptors enhances proliferation at low ligand levels, as well as inducing Müller cell differentiation prematurely at higher ligand levels (L.L., manuscript in preparation). By the time of birth, levels of EGF-R expressed by retinal progenitor cells normally may be saturating for the generation of mitotic responses but not for effects on cell fate. Müller cell differentiation begins primarily during the first postnatal week, a time when progenitor cell proliferation is also declining^{1,21}. Müller cell differentiation may normally be achieved by increased expression of EGF-Rs in some progenitor cells during the first postnatal week, and/or exposure of a subpopulation of progenitor cells to high local concentrations of ligand. Consistent with this hypothesis, heterogeneity in the level of endogenous EGF-R expression was observed during the first postnatal week (Fig. 2). The introduction of extra EGF receptors could serve prematurely to bring more cells to threshold levels of receptor activation to support Müller cell development in response to lower concentrations of ligand. Although TGF- α has direct effects on differentiation^{22–24} and its effects on Müller cell differentiation do not appear to involve a stimulatory effect on proliferation, whether higher levels of EGF-R activation affect differentiation directly, or act through inhibitory effects on cell-cycle progression, remains to be resolved.

The findings presented here suggest that signalling mediated by EGF-Rs can affect the choice of postnatal retinal progenitor cells to adopt a glial versus a neuronal/photoreceptor fate. A similar role for GGF in the regulation of neural crest cell development into glial cells was reported recently²², and may reflect a general function of this growth factor family. Observations presented in this study indicate that limitations in both ligand availability and the level of receptor expression can contribute to the regulation of this choice in the retina. Moreover, these studies demonstrate that the introduction of extra receptors *in vivo* can overcome limitations in receptor expression to influence cell fate. □

Received 7 April; accepted 11 July 1995.

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ACKNOWLEDGEMENTS. I thank D. Mason for BrdU antibody, C. Barnstable for Ret P1 antibody and D. Lowy for pC011-EGFR-neo. The pLIGS vector was made from a vector (pLIGI) made in the laboratory of C. Cepko. I thank D. Wancio for technical assistance, N. Cook and K. Engisch for assistance with confocal microscopy, J. Sparrow for advice on cell type markers, and N. Bonini, K. Engisch, I. Fischer, W. Harris, P. Levitt, M. Nowicky and M. Raff for their valuable comments on the manuscript. This work was supported by a Basil O'Connor Starter Scholar Research Award from the March of Dimes.

High susceptibility to ultraviolet-induced carcinogenesis in mice lacking XPC

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COMPROMISE of genetic information by mutation may result in the dysregulation of cellular growth control and subsequent tumour formation. Xeroderma pigmentosum (XP) is a rare autosomal disease characterized by hypersensitivity of the skin to sunlight and >1,000-fold increased risk of skin cancers in sun-exposed parts of the body. Cell fusion studies have revealed eight complementation groups in XP (A–G, and an XP-variant form); group C is one of the most common forms of the disease¹. We have isolated a mouse homologue of the human gene for XP group C and generated *XPC*-deficient mice by using embryonic stem cell technology. Mice homozygous for the *XPC* mutant allele (*xpc^{m1}/xpc^{m1}*) are viable and do not exhibit an increased susceptibility to spontaneous tumour generation at one year of age. However, *xpc^{m1}/xpc^{m1}* mice were found to be highly susceptible to ultraviolet-induced carcinogenesis compared with mice heterozygous for the mutant allele (*xpc^{m1}/+*) and wild-type controls. Homozygous *xpc^{m1}* mutant mice also display a spectrum of ultraviolet-exposure-related pathological skin and eye changes consistent with the human disease xeroderma pigmentosum group C.

Cells cultured from XP patients have an increased sensitivity to ultraviolet radiation, a phenotype related to defective nucleotide excision repair (NER)². The human *XPC* gene was previously cloned by complementation of the NER defect and

contains a region of homology to the yeast *RAD4* gene³. We have cloned the mouse homologue of *XPC* from a mouse 129-strain genomic library by hybridization with a human *XPC* probe. Mouse *XPC* has extensive sequence similarity to human *XPC*: 78% identity was found at the nucleotide level and 79% identity at the amino-acid level between mouse exonic regions and the human complementary DNA sequence (Fig. 1a). To

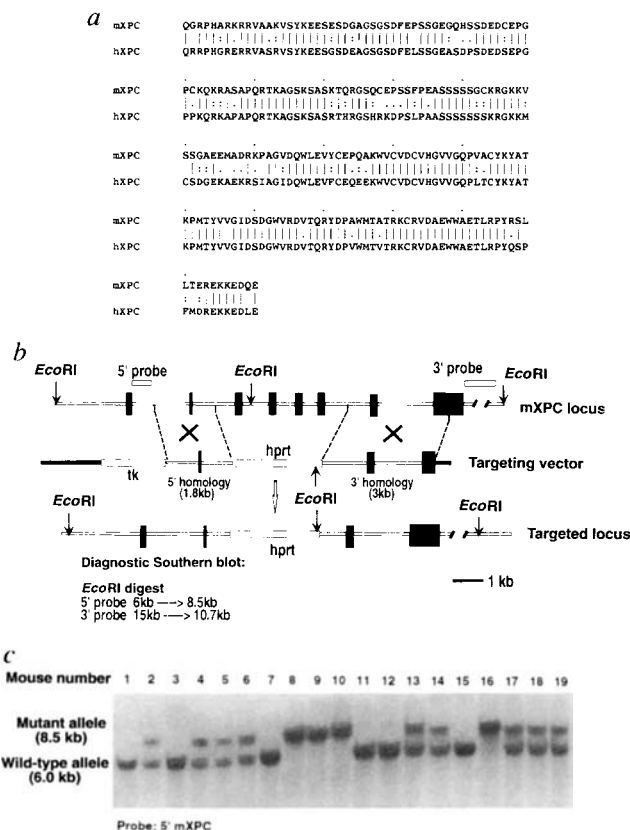


FIG. 1 Cloning and gene targeting of a mouse homologue of XPC. a, Comparison of partial deduced amino-acid sequence of mouse XPC (mXPC) and human XPC (hXPC). The deduced amino-acid sequence from part of a single large exon in the mouse *XPC* genomic locus was compared with a human *XPC* amino-acid sequence. b, Targeting of *XPC* genomic locus. A replacement targeting vector containing 1.8 kb of 5' homology and 3 kb of 3' homology was constructed with a marker for positive selection (*hprt* mini-gene)¹³ and negative selection⁴ (thymidine kinase). c, Southern blot analysis of genomic DNA from offspring of *xpc^{m1}/+* intercross. The presence of a single 6-kb fragment in a lane indicates a wild-type animal, a single 8.5-kb fragment represents an animal homozygous for the targeted mutant allele (*xpc^{m1}/xpc^{m1}*), and both fragments in a lane indicate a heterozygous animal (*xpc^{m1}/+*). METHODS. Deduced amino-acid comparison was performed with the GAP program in GCG sequence analysis package, version 8, Genetics Computer Group, Madison, Wisconsin. Identical amino acids are indicated by a vertical line between the mouse and human sequences, conservative amino acid changes are shown with two dots, and moderately conservative amino acid changes are shown with one dot, using threshold values of 1.5, >0.5 and >0.1 respectively¹⁴. The targeting vector was transfected into AB 2.1 mouse ES cells¹³, selection applied, and resultant colonies screened by minisouthern as previously described¹⁵. Ten positive clones were identified as correct replacement events with an approximate 4.5-kb genomic deletion as indicated using both 3' and 5' external probes (targeting frequency approximately 1/20). Two targeted ES cell clones were injected into host blastocysts to form chimeric mice, one of which transmitted the mutant allele through the germline as determined by Southern blot analysis. F₁ progeny heterozygous for the mutant allele were mated and genomic DNA was isolated from the progeny. Genomic DNA was digested with restriction endonuclease *EcoRI* and a Southern blot performed with a 5' probe for the mouse *XPC* locus external to the region of homology in the target construct.

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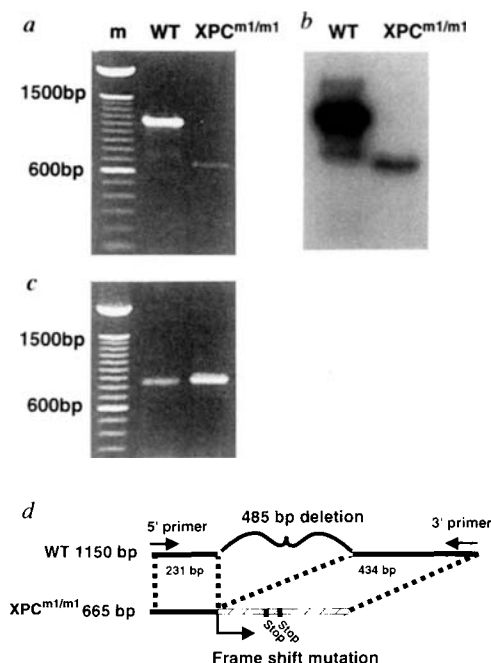


FIG. 2 RT-PCR on message from wild-type and *xpc*-deficient cells. **a**, RT-PCR for *XPC* message on RNA from *xpc*^{m1/xpc}^{m1} (*xpc*^{m1/m1}) and wild-type (WT) embryonic fibroblasts reveals a predicted 1,150-bp fragment with wild-type RNA as template and a 665-bp fragment with RNA from mutant cells. **b**, A Southern blot of the gel pictured in **a** hybridized with an exonic *XPC* probe that was outside the deleted region indicated that wild-type and mutant RT-PCR product recovered was, in fact, derived from *XPC* message. **c**, RT-PCR-positive controls using primers to the *Mgf* gene¹⁶ indicated that reaction conditions were optimized for both wild-type and mutant RNA templates. **d**, Summary of sequencing results of mutant and wild-type bands reveals a 485-bp deletion in the mutant message resulting in a frameshift mutation and stop codons 151 bp downstream from the deletion point.

METHODS. mRNA was isolated from wild-type and *xpc*^{m1/xpc}^{m1} mouse embryonic fibroblasts (QuickPrep Micro mRNA purification kit, Pharmacia). 1.2 µg of mRNA was used to make 1st-strand cDNA using oligo(dT) primers (Superscript RT II kit, Gibco BRL). Primer sequence was as follows: *XPC* 5' primer, GCT GCA GTG ATC CAG GGG AC; *XPC* 3' primer, TGG ACG GTT CAA AGT CAG AG. *Mgf* 5' primer, AGC TGC AGC TGG ATC GCA GCG CTG CCT TTC C; *Mgf* 3' primer, TCG AGC TCT GTT GAT ACG TCC ACA ATT ACA CCT C. PCR conditions were 93 °C for 40 s, 72 °C for 90 s; 34 cycles. PCR products were subcloned into a T-Vector (Promega) and sequenced.

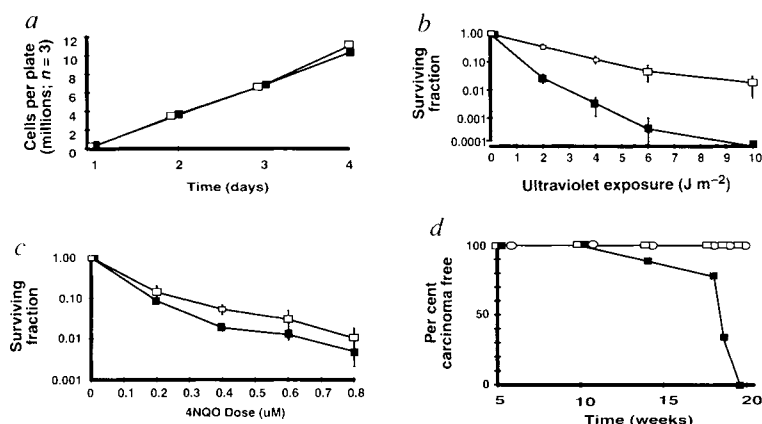
generate *XPC*-deficient mice, we used a replacement vector construct with positive and negative selection⁴ to delete ~4.5 kilobases (kb) of the mouse *XPC* genomic locus by homologous recombination in embryonic stem (ES) cells. Targeted clones with the predicted replacement event were identified with probes both 5' to the homology region (Fig. 1b) and 3' to the homology region (results not shown). This deletion eliminated a coding portion of the mouse gene corresponding to bases +59 to +546 of the published human *XPC* cDNA³. Targeted ES cells were injected into host blastocysts to generate chimaeric animals which transmitted the mutant allele (*xpc*^{m1}) to F₁ offspring (C57BL/129 hybrids) as identified by Southern blot analysis. Of 150 offspring from *xpc*^{m1/+} intercrosses, 25% were wild-type, 46% *xpc*^{m1/+} and 29% *xpc*^{m1/xpc}^{m1}, approximating the expected mendelian ratios and indicating that there is no developmental

selection against the mutant allele (Fig. 1c). Homozygous *xpc*^{m1} mutant mice are fertile.

To characterize the effect of the deletion mutation on the *XPC* message, RNA was isolated from wild-type and *xpc*^{m1/xpc}^{m1} primary embryonic fibroblasts reverse transcribed and amplified by the polymerase chain reaction (RT-PCR). By using primers to exons flanking the deleted region, the predicted 1,150 base-pair (bp) fragment (Fig. 2) was obtained with wild-type RNA as a template (a faint 750-bp fragment in the wild-type lane was also detected, which may represent an alternatively spliced product). A 1,150-bp fragment was not produced from the RT-PCR reaction on RNA from *xpc*^{m1/xpc}^{m1} mutant cells; instead, a smaller 665-bp fragment of much lower intensity was present (Fig. 2a). Both fragments hybridized to an *XPC* exonic probe outside the deleted region (Fig. 2b). The wild-type and

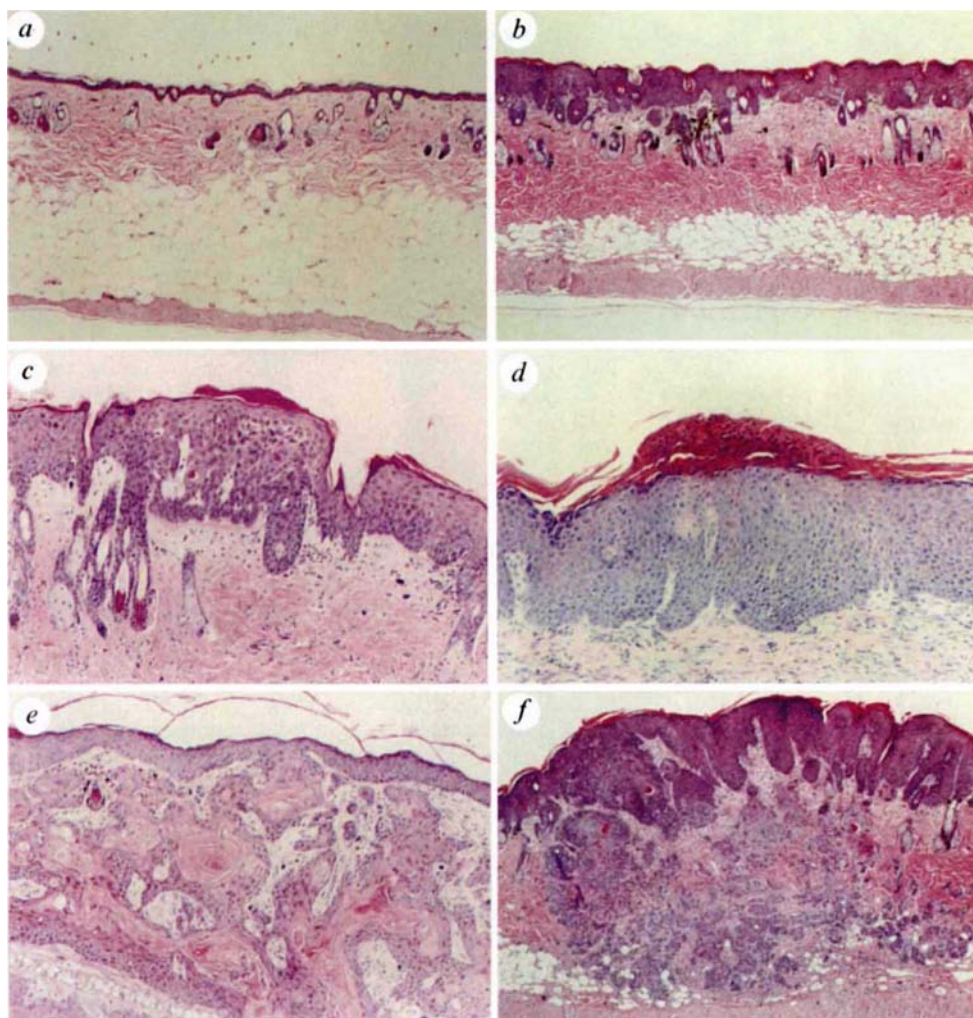
FIG. 3 DNA damage and carcinogenesis in *XPC* deficiency. **a**, *xpc*^{m1/xpc}^{m1} (filled squares) and wild-type (open squares) primary embryonic fibroblasts proliferate at roughly equal rates in cell culture. **b**, Ultraviolet irradiation colony survival assay was performed on *xpc*^{m1/xpc}^{m1} (filled squares) and wild-type (open squares) primary embryonic fibroblasts. *xpc*^{m1/xpc}^{m1} cells show significantly decreased ability to form colonies after exposure to ultraviolet at several dose points compared with their wild-type counterparts ($P < 0.01$, bars represent standard deviation). **c**, 4NQO colony survival assay of *xpc*^{m1/xpc}^{m1} and wild-type cells. *xpc*^{m1/xpc}^{m1} cells (filled squares) demonstrate no statistically significant (t -test) decreased ability to form colonies after exposure to the genotoxic agent 4NQO compared with wild-type cells (open squares). **d**, Time to tumour formation was measured in *XPC*-deficient mice with chronic exposure to ultraviolet. *xpc*^{m1/xpc}^{m1} (filled squares, $n = 9$) mice showed a rapid induction of skin carcinomas after daily exposure to ultraviolet compared with *xpc*^{m1/+} (open circles, $n = 8$) and wild-type (open squares, $n = 8$) littermates. All tumours observed were confirmed by histopathological analysis.

METHODS. Primary embryonic fibroblasts were isolated from 13.5-day embryos derived from a cross of *xpc*^{m1/+} mice (C57BL/129 hybrids). Three cell lines of each genotype were plated at low density and exposed to increasing doses of ultraviolet. After exposure, cells were incubated for 10 days in DMEM, 15% fetal calf serum, and 40% pre-conditioned media with antibiotics to allow colony formation. Colonies were then fixed and stained; those <50 cells were counted. The average number of colonies per plate of three independent cell lines was calculated for each ultraviolet dose; standard deviations (bars) and statistical



significance were determined (t -test). For the carcinogenesis study, mice 8 to 12 weeks old (C57BL/129 hybrids) were exposed to an average of 2,500 J m⁻² per day (maximal emission in the UV-B wavelength range) for a 20-week period. All mice were shaved once per week during the 20-week exposure period and were regularly observed for pathological changes in the skin. Lesions that were identified as tumours grossly were noted and histopathological confirmation of carcinomas was obtained when animals were killed at the end of the 20-week ultraviolet treatment.

FIG. 4 Histological appearance of lesions induced in xpc^{m1}/xpc^{m1} mice by ultraviolet radiation. a, Dorsal skin of an irradiated wild-type mouse showing uniform epidermal hyperplasia and thickening of the dermis (magnification $\times 25$). b, Dorsal skin of xpc^{m1}/xpc^{m1} irradiated mouse ($\times 25$) showing a severe epidermal hyperplasia, thickening and fibrosis of the dermis. c, Area of focal hyperplasia with dysplasia and epidermolysis from the dorsal skin of an irradiated xpc^{m1}/xpc^{m1} mouse ($\times 50$). d, Carcinoma *in situ* from the dorsal skin of an irradiated xpc^{m1}/xpc^{m1} mouse ($\times 50$). e, Well differentiated squamous cell carcinoma from the ear of an irradiated xpc^{m1}/xpc^{m1} mouse ($\times 25$). f, Moderately differentiated squamous cell carcinoma from the dorsal skin of an irradiated xpc^{m1}/xpc^{m1} mouse ($\times 25$). METHODS. Tissue specimens were placed in neutral buffered formalin and embedded in paraffin. 5- μ m sections were stained with haematoxylin and eosin by conventional methods.



mutant bands were sequenced and confirmed to correspond to *XPC* message (results not shown). Sequencing the mutant fragment revealed a 485-bp deletion yielding a frameshift mutation and two stop codons 151 nucleotides downstream of the deletion (Fig. 2c). This mutant message results from the deletion of *XPC* exons in the targeted allele and transcript splicing around the hypoxanthine phosphoribosyl transferase mini-gene¹³.

XPC-deficient embryonic fibroblasts proliferated in culture as rapidly as wild-type cells, and their plating efficiencies were similar (Fig. 3a). On exposure to ultraviolet light, however, xpc^{m1}/xpc^{m1} cells showed a significantly decreased ability to form colonies compared with wild-type cells (Fig. 3b). Embryonic fibroblasts heterozygous for the mutant allele could not be distinguished from wild-type cells in this assay (results not shown). Increased sensitivity of xpc^{m1}/xpc^{m1} fibroblasts to 4-nitroquinoline 1-oxide (4NQO), a known mutagenic agent that acts principally at guanine residues⁵, was not found to be statistically significant at the dose points tested (Fig. 3c). *XPC* deficiency in these mouse primary cell lines therefore significantly altered cell survival in response to the type of damage caused by ultraviolet but not that associated with 4NQO exposure. This finding differs from previous reports of increased 4NQO sensitivity of human *XP* cell lines^{6,8}.

To measure the *in vivo* sensitivity to ultraviolet-induced tumour formation, xpc^{m1}/xpc^{m1} ($n=9$), $xpc^{m1}/+$ ($n=8$), and wild-type ($n=8$) litter mates were exposed daily to ultraviolet. After 10 days of treatment, all xpc^{m1}/xpc^{m1} mice showed marked atrophy of the ears whereas $xpc^{m1}/+$ and wild-type animals appeared normal. Treatment was continued for 20 weeks, by the end of which all homozygous xpc^{m1} mice had developed at least

one skin carcinoma, confirmed by histological sections (Fig. 3d). The predominant location of tumour formation was the ears, and some mice developed multiple tumours. A histopathological survey of tumour types revealed predominantly well and moderately differentiated squamous cell carcinomas (Fig. 4e, f). Mice heterozygous for the mutant *XPC* allele and wild-type controls did not develop any tumours by the end of the treatment (Fig. 4a). In addition to these neoplastic lesions, ultraviolet-irradiated xpc^{m1}/xpc^{m1} mice showed marked hyperplasia of the epidermis (Fig. 4b) with focal areas of hyperkeratosis and varying degrees of dysplasia, acantholysis and/or dyskeratosis (Fig. 4c, d). These lesions have been previously described in ultraviolet-irradiated hairless mice⁹ and are similar to the human lesion known as actinic or solar keratosis^{10,11}. Ultraviolet treatment also produced frequent abnormalities in eyes of xpc^{m1}/xpc^{m1} mice including severe keratitis and corneal ulceration, which were not found in wild-type and heterozygous animals. A colony of xpc^{m1}/xpc^{m1} , $xpc^{m1}/+$ and wild-type mice ranging from 10 to 14 months of age that were not exposed to ultraviolet irradiation did not show any signs of tumour formation or any other pathological changes.

We have cloned the mouse homologue of human *XPC* and produced mice with a deletion mutation in this gene by using ES cell technology. The mutant allele results in a message with a deletion, frameshift and stop codons. The mutation is located earlier in the coding region than any of the mutations described so far in cell lines derived from *XPC* patients¹², suggesting that this allele is functionally null, although we cannot exclude the possibility that an amino-terminal peptide with limited activity might be produced. Embryonic fibroblasts homozygous for the

mutant allele show a marked sensitivity to ultraviolet consistent with the sensitivity of cells derived from XPC patients¹. Mice homozygous for the mutant allele, like XPC patients, are highly susceptible to skin carcinomas after ultraviolet exposure and display a wide range of ultraviolet-induced histopathological skin changes. □

Received 24 March; accepted 7 August 1995.

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High incidence of ultraviolet-B- or chemical-carcinogen-induced skin tumours in mice lacking the xeroderma pigmentosum group A gene

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XERODERMA pigmentosum (XP) is an autosomal recessive disorder characterized by a high frequency of skin cancer on sun-exposed areas, and neurological complications. XP has a defect in the early step(s) of nucleotide-excision repair (NER) and consists of eight different genetic complementation groups (groups A–G and a variant)¹. We established *XPA* (group-A XP) gene-deficient mice by gene targeting of mouse embryonic stem (ES) cells. The *XPA*-deficient mice showed neither obvious physical abnormalities nor pathological alterations, but were defective in NER and highly susceptible to ultraviolet-B- or 9,10-dimethyl-1,2-benz[*a*]anthracene-induced skin carcinogenesis. These findings provide *in vivo*

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ACKNOWLEDGEMENTS. We thank S. Jackson for taking care of the mice in this study; and also E. Gimenez-Conti for histological services at Science Park, University of Texas M.D. Anderson, Smithville. This work was partly supported by DHS (C.C.). A.S. is an American Cancer Society Postdoctoral Fellow. A.B. is a Howard Hughes Investigator and this work was partly supported by a grant from NICHD.

evidence that the *XPA* protein protects mice from carcinogenesis initiated by ultraviolet or chemical carcinogen. The *XPA*-deficient mice may provide a good *in vivo* model to study the high incidence of skin carcinogenesis in group A XP patients.

We cloned the *XPA* gene and its mouse homologue^{2,3}, which may play important roles in damage recognition^{4,6} and coordination of NER processes by interacting with other proteins^{7–10}. To elucidate the *in vivo* function and molecular basis of the group-A XP phenotype, we established *XPA*-deficient mice. Mice with a disrupted *XPA* allele were generated by insertion of neomycin (*neo*) cassette sequences into exon 4 of the *XPA* gene by using ES cell techniques¹¹ (Fig. 1*a–c*). The heterozygous (+/–) mice did not display any abnormal phenotype compared with their wild-type (+/+) littermates as judged by physical observation. The (+/–) mice were intercrossed to obtain homozygous (–/–) mice. The frequencies of the weaned pups with (+/+), (+/–) and (–/–) *XPA* genotypes were 24.5% (179/729), 49.8% (363/729) and 25.7% (187/729) respectively, indicating that the disruption of the *XPA* gene by our method does not result in embryonic lethality. The (–/–) mice showed neither obvious physical abnormalities nor pathological alterations (results not shown) and were fertile. To confirm that no *XPA* gene products were being synthesized in the (–/–) mice, western blot analysis of the splenocyte extracts with anti-mouse *XPA* protein antibody was performed. About half of the *XPA*

TABLE 1 UV-B-induced skin carcinogenesis in *XPA* (–/–) mice

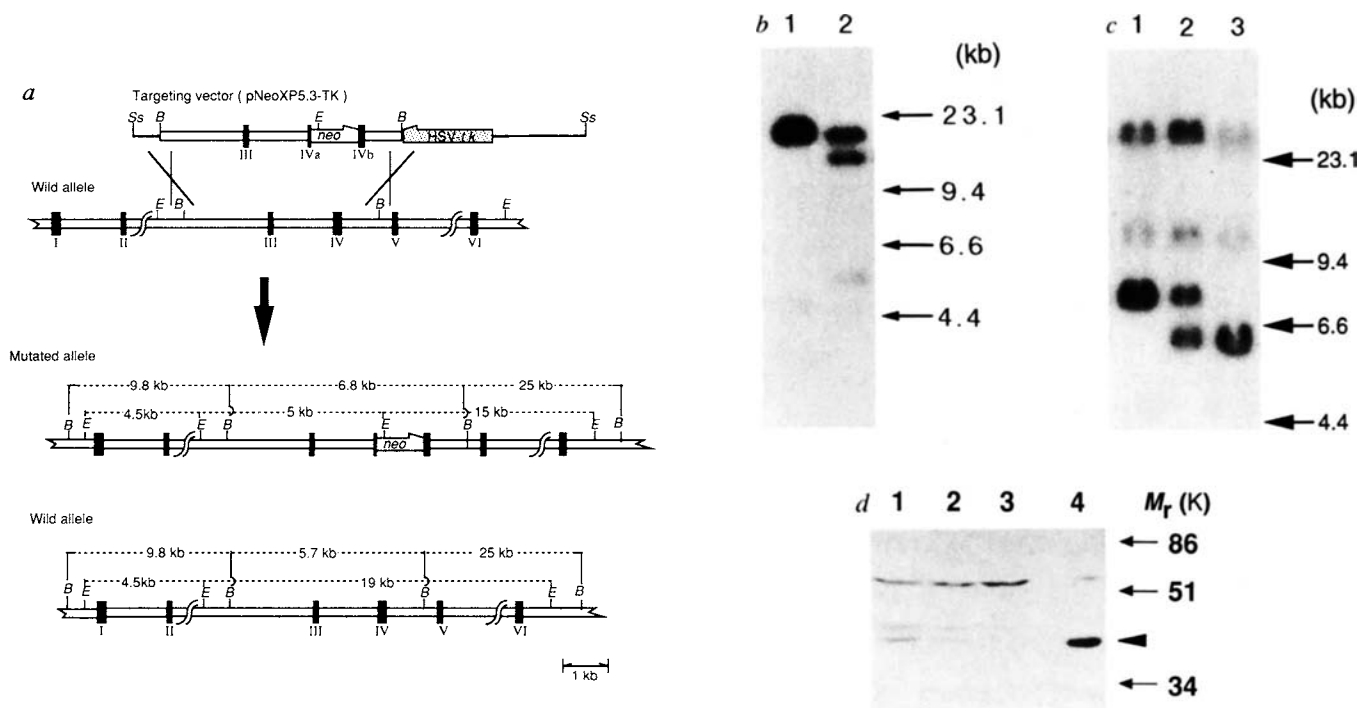
Mouse	Weeks of irradiation at detection of first tumour	Histological diagnosis	Tumour formation in nude mice†
1	30	SCC	(+)
2	20	Died at 37 weeks*	NT
3	27	SCC	(+)
4	26	SCC	(+)
5	32	SCC	(+)
6	21	Died at 26 weeks*	NT
7	22	SCC	(+)
8	31	SCC	(+)
9	26	SCC	(+)
10	23	Died at 26 weeks*	NT
11	27	SCC	—
12	32	SCC	—
13	29	SCC	(+)
14	34	SCC	(+)
15	29	SCC	(+)

* Died of rapidly growing tumours before histological analysis.

† Primary tumours were cut into 1-mm³ fragments and implanted subcutaneously with a trocar in one flank of 4–8 nude mice. The recipients were inspected weekly. Progressively growing tumours were defined as positive for tumour generation 4 weeks after transplantation. No progressively growing tumours were detected in recipients from mice 11 and 12 after 4 weeks of observation. NT, not tested.

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METHODS. **a**, For construction of a targeting vector, a 5.7-kb *Bam*HI *XPA* genomic fragment containing exons 3 and 4 was subcloned into a pUC19 plasmid. The 1.1-kb *Xho*I–*Bam*HI *neo* cassette from pMC1 *neo*/poly (A) (Stratagene) was inserted in the same transcriptional orientation into the *Eco*NI site of exon 4. For positive–negative selection¹³, the herpes simplex virus *tk* (HSV-*tk*) gene was inserted in a reverse-transcriptional orientation into the *Sal*I site of pUC19 on the 3' side of the *XPA* sequences (pNeoXP5.7-TK). *Ssp*II-digested pNeoXP5.7-TK was electroporated into F1/1 ES cells derived from the F₁ hybrid of CBA and C57BL/6 (ref. 14) using a Bio-Rad Gene Pulser. The cells were then incubated for 48 h on feeder layers of G418-resistant STO cells. After 10 days of culture in medium containing 150 µg ml^{–1} G418 (Gibco) and 2 µM gancyclovir (GANC; Syntex Research), individual colonies were isolated and DNA was extracted for polymerase chain reaction (PCR) to identify *XPA* homologous recombinants. **b**, Genomic DNA from PCR-positive ES cell clones was digested with *Eco*RI, blotted and hybridized with mouse *XPA* complementary DNA³. **c**, The homologous recombinant ES cell clones were injected into 8-cell embryos of CD-1. The embryos were then implanted into the uteri of pseudopregnant CD-1 foster mothers, generating 14 chimaeras as judged by the presence of agouti coat-colour pigmentation. Four chimaeric males were bred against CD-1 females and a male chimaera with about 100% contribution of ES cells with respect to coat colour showed germline transmission. To diagnose the *XPA* genotype of the offspring, genomic DNA from the mouse tail tip was digested with *Bam*HI, blotted and hybridized with mouse *XPA* cDNA. About 70% of the offsprings carried the modified *XPA* allele. The (+/–) mice were intercrossed to obtain the (–/–) mice. **d**, Western blot analysis was done as described⁷. The antibody was elicited in rabbits by using recombinant mouse *XPA* protein, lacking the amino-terminal 23 amino acids, as an antigen.

protein detected in the (+/+) mice was produced in the (+/–) mice, whereas no *XPA* proteins were detected in the (–/–) mice (Fig. 1d).

To confirm that the targeting of the *XPA* gene disrupted the NER capacity of mice, the colony-forming abilities of fibroblasts derived from (–/–), (+/–) and (+/+) newborn mice were compared after various doses of ultraviolet (UV) (254 nm) irradiation. As shown in Fig. 2A, the (–/–) fibroblasts were hypersensitive to UV irradiation, whereas the (+/–) and (+/+) fibroblasts were resistant. The UV survival curve of fibroblasts from (–/–) mice was almost the same as that from group-A XP patients with a typical clinical phenotype ($D_{37} = 0.3 \text{ J m}^{-2}$ for the (–/–) mouse fibroblasts; $0.2\text{--}0.4 \text{ J m}^{-2}$ for the cells from group-A XP patients; D_{37} is the dose for 37% survival). Repair synthesis of DNA during NER (unscheduled DNA synthesis (UDS)) in the (–/–), (+/–) and (+/+) fibroblasts was 3 ± 2 ,

61 ± 14 and 58 ± 12 (mean grain number ± s.d.), respectively. Thus the UV-induced UDS was absent in the (–/–) fibroblasts. We also compared the kinetics of removal of major UV-induced DNA lesions in these fibroblasts by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies against cyclobutane-type thymine dimers and (6-4) photoproducts. Neither thymine dimers nor (6-4) photoproducts were removed in the (–/–) fibroblasts but both were removed in the (+/+) and (+/–) fibroblasts (Fig. 2B). Thus we concluded that the (–/–) mouse was defective in NER, similarly to typical human group-A XP patients. To confirm that the defective NER in the (–/–) fibroblasts was due to a disruption of the *XPA* gene, we performed a genetic complementation analysis between group A (XP6TO) or C (GM2096) XP cells and (–/–) fibroblasts. The fused binuclear cells, showing a normal level of UV-induced UDS, were detected in the combination of group-C XP cells and

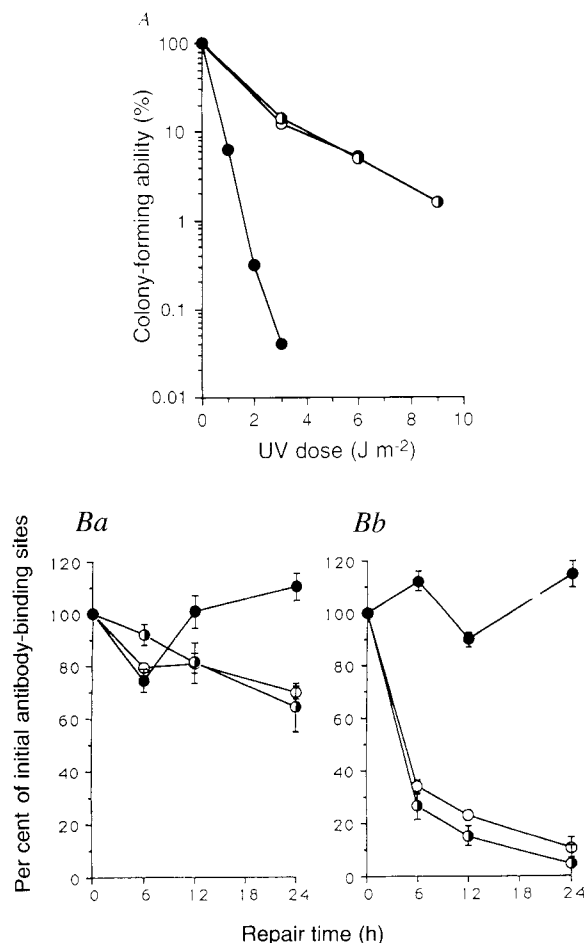
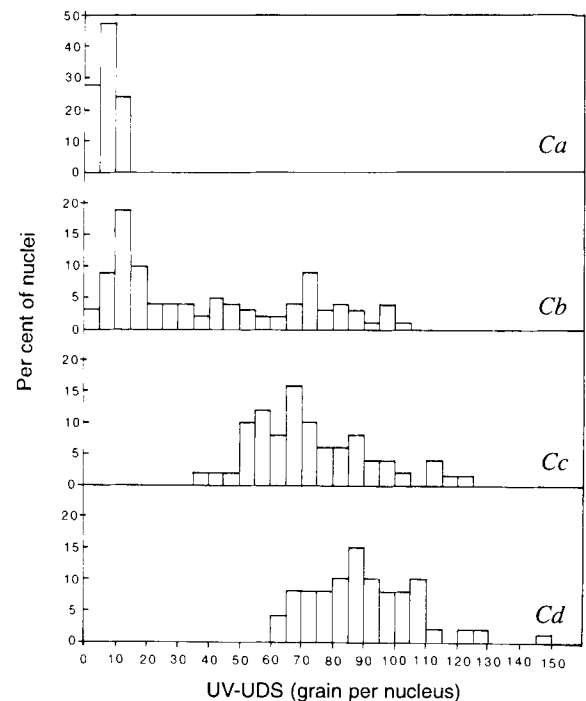


FIG. 2 NER capacity of the XPA-deficient mice. **A**, UV survival curves of fibroblasts derived from (+/+) (open circles), (+/-) (half-filled circles) and (-/-) (filled circles) newborn (1-day) mice. **B**, Removal of thymine dimers and (6-4) photoproducts in fibroblasts derived from the (+/+), (open circles), (+/-), (half-filled circles) and (-/-) (filled circles) newborn mice. **Ba**, removal of thymine dimers after 10 J m^{-2} UV irradiation;

(-/-) fibroblasts, but were not detected in the combination of group-A XP cells and (-/-) fibroblasts (Fig. 2C). These findings indicate that the XP genotype of the (-/-) mice was the same as that in the group-A XP patients, and that the defective NER in the (-/-) mice is caused by a disruption of the *XPA* gene.

Next we examined the susceptibility of the mice to UV. As shown in Fig. 3A, a single ultraviolet-B (UV-B; 280–320 nm) irradiation induced more severe inflammatory oedema in the ears of (-/-) mice than in those of (+/+) and (+/-) mice, indicating that (-/-) mice are highly susceptible to UV-B. We then examined UV-B-induced skin carcinogenesis in these mice. The shaved back skins of 15 (-/-), 22 (+/-) and 15 (+/+) mice were irradiated three times a week with UV-B at a dose of 2 kJ m^{-2} per exposure. Areas other than shaved back skin were shielded from UV-B. After 1–2 weeks of UV-B irradiation, all the (-/-) mice developed skin ulcers in the exposed areas, also indicating that the (-/-) mice are highly susceptible to UV-B. The ulcers were gradually surrounded by poorly defined areas of hyperkeratotic adherent scale. After 20 weeks of UV-B irradiation, the first skin tumour was detected in the (-/-) mice as a nodule in the area of the hyperkeratotic scale, and by 34 weeks of UV-B irradiation all (-/-) mice had developed such tumours (Table 1). Three (-/-) mice (nos 2, 6 and 10) died of rapidly growing tumours after 37, 26 and 26 weeks of UV-B irradiation. On the other hand, the (+/+) and (+/-) mice did not develop any significant skin abnormalities during the UV-B-irradiation experiments. All the (-/-), (+/-) and (+/+) mice



Bb, removal of (6-4) photoproducts after 10 J m^{-2} UV irradiation. Bars represent the standard error of the mean where the points (symbols as in **A**) are the average values. **C**, Genetic complementation analysis of the (-/-) fibroblasts. **Ca**, UV-induced UDS in binuclear cells obtained from combining the (-/-) fibroblasts and group-A XP cells (XP6TO); **Cb**, binuclear cells obtained from combining the (-/-) fibroblasts and group-C XP cells (GM2096); **Cd**, homodikaryons of (+/+) fibroblasts; **Ce**, homodikaryons of normal human fibroblasts (FS3). The mean number of grains per nucleus (calculated from 100 nuclei) in the homodikaryons of group-A XP (XP6TO), group-C XP (GM2096) and (-/-) mouse cells were 3.3, 7.2 and 2.2, respectively.

METHODS. **A**, UV survival was determined as described². **B**, The rate of removal of thymine dimers and (6-4) photoproducts was measured by EISA using the monoclonal antibodies TDM-1 (specific for thymine dimers) and 64M-2 (specific for (6-4) photoproducts)¹⁵. **C**, Genetic complementation analysis was performed as described¹⁶.

mice were killed for histological analysis at week 39. All of the skin tumours in the (-/-) mice were histologically diagnosed as squamous cell carcinoma (SCC; Table 1, Fig. 3B). So far, 10 out of 12 primary skin tumours from the (-/-) mice have developed progressively growing tumours when transplanted into nude mice (Table 1), confirming their malignant characteristics. On the other hand, the UV-B-irradiated skins of (+/-) and (+/+) mice were nearly normal (Fig. 3B). These findings indicate that the *XPA* (-/-) mice are highly susceptible to UV-B-induced skin carcinoma.

We also analysed a chemical carcinogen, 9,10-dimethyl-1,2-benz[*a*]anthracene (DMBA), with regard to inducing skin tumours in these mice. Groups of 21 (-/-), 14 (+/-) and 14 (+/+) mice were repeatedly treated with $10 \mu\text{g}$ DMBA at weekly intervals. The first tumours were observed after 6, 14 and 14 weeks of DMBA treatment in the (-/-), (+/-) and (+/+) mice, respectively, and the percentages of tumour-bearing mice at week 18 increased to 90, 28 and 21% in the (-/-), (+/-) and (+/+) mice, respectively (Fig. 3C). Tumours were histologically diagnosed as papillomas (Fig. 3D). The differences in the percentages of tumour-bearing mice between (-/-) and (+/-) mice at week 18 were statistically significant ($P < 0.01$). The average numbers of papillomas per mouse at week 18 were 4.8 (-/-), 0.4 (+/-) and 0.2 (+/+), the differences between (-/-) and (+/-) mice being statistically significant ($P < 0.05$). Thus the *XPA* (-/-) mice are also highly susceptible to DMBA-induced skin tumour formation.

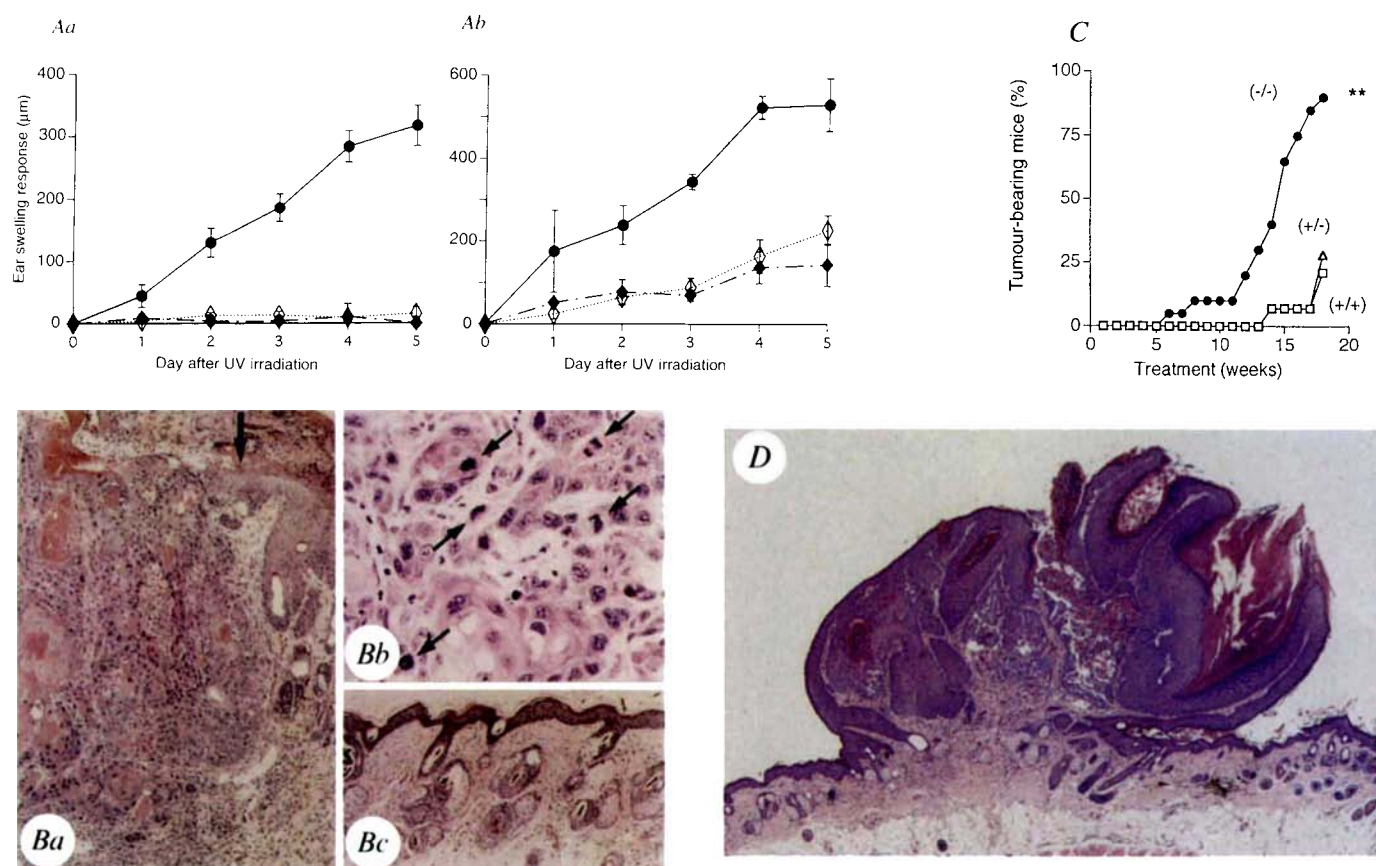


FIG. 3 UV-B- or DMBA-induced skin tumour formation. **A**, Acute inflammatory oedema in the ears of UVB-irradiated mice. UVB doses are: **Aa**, 1 kJ m^{-2} ; **Ab**, 5 kJ m^{-2} . Symbols used are: filled circles, (-/-); filled diamonds, (+/-); open diamonds (+/+) mice. **B**, Histological sections of UV-B-induced tumour in the (-/-) mouse and nearly normal skin in the UV-B-irradiated (+/+) mouse. **Ba**, A well differentiated squamous cell carcinoma invading into the dermis in the skin of a UV-B-irradiated (-/-) mouse. Magnification, $\times 46$. Note the non-carcinoma epidermis on the right-hand side of the arrow. **Bb**, A higher magnification ($\times 200$) of carcinoma cells. Arrows indicate mitotic figures. **Bc**, Nearly normal skin tissue of a UV-B-irradiated (+/+) mouse. Magnification, $\times 46$. **C**, Time course of skin-tumour formation during repeated treatment with a low dose of DMBA. Symbols used are: filled circles, (-/-); open triangles, (+/-), and open squares, (+/+) mice. A significant difference in the percentage of tumour-bearing mice was observed between (-/-) and (+/+) mice (**, $P < 0.01$) by the χ^2 test. **D**, Histological section of DMBA-induced skin papilloma in a (-/-) mouse. Magnification, $\times 26$.

All these findings indicate that the XPA protein protects mice from carcinogenesis initiated by UV or chemical carcinogen, and that XPA-deficient mice may provide a useful model to study the high incidence of skin carcinogenesis in group-A XP. Many group-A XP patients develop several neurological complications such as microcephaly, progressive mental deterioration and sensorineural deafness. The major patho-

METHODS. **A**, UV-B irradiation and measurement of ear swelling were performed as described¹⁷. Each experimental and control group consisted of 5 mice (10 ears). **B**, Back skin of female (15 (-/-), 22 (+/-) and 15 (+/+)) mice, 8 weeks old, with agouti or black coat colour was shaved with electric clippers and irradiated three times a week (on Mondays, Wednesdays and Fridays) with UV-B at a dose of 2 kJ m^{-2} per exposure. Areas other than shaved back skin were shielded from UV-B by putting the mouse into an acrylic resin box with a window during the irradiation. Mice were killed after 39 weeks of UV-B irradiation, and representative organs including skin tissues were examined histopathologically. Sections were stained with haematoxylin and eosin. **C**, Shaved back skin of female (14 (+/+), 14 (+/-) and 21 (-/-)) mice, 8 weeks old, were painted with $10 \mu\text{g}$ of 9,10-dimethyl-1,2-benz[a]anthracene (DMBA; Wako Pure Chemical Industries, Osaka, Japan) in 0.2 ml acetone once a week for 18 weeks. Scoring for tumours was done once a week. **D**, All the mice were killed at 18 weeks and representative organs including the skin were examined histopathologically.

logical findings in the nervous system of group-A XP patients are loss of neurons and neurodegeneration, particularly in the cerebral cortex and cerebellum¹². But pathological analysis of neural tissues from the XPA-deficient mice revealed no neurodegeneration or gliosis up to 18 months of age (results not shown). The reason for this difference is not yet known. □

Received 30 June; accepted 31 July 1995.

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ACKNOWLEDGEMENTS. H.N., S.T. and S.Y. contributed equally to this work. We thank M. Katsuki and H. Takahashi for discussion about analysing the mice, and Y. Yamazaki for technical assistance. S.Y. was supported by postdoctoral fellowships in cancer research of the Japan Society for the Promotion of Science.

Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene *XPA*

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XERODERMA pigmentosum patients with a defect in the nucleotide-excision repair gene *XPA* are characterized by, for example, a >1,000-fold higher risk of developing sunlight-induced skin cancer¹⁻³. Nucleotide-excision repair (NER) is involved in the removal of a wide spectrum of DNA lesions. The *XPA* protein functions in a pre-incision step, the recognition of DNA damage⁴⁻⁷. To permit the functional analysis of the *XPA* gene *in vivo*, we have generated *XPA*-deficient mice by gene targeting in embryonic stem cells. The *XPA*^{-/-} mice appear normal, at least until the age of 13 months. *XPA*^{-/-} mice are highly susceptible to ultraviolet (UV)-B-induced skin and eye tumours and to 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced skin tumours. We conclude that the *XPA*-deficient mice strongly mimic the phenotype of humans with xeroderma pigmentosum.

A deletion spanning exons 3 and 4 was introduced in the mouse *XPA* gene by homologous recombination in embryonic stem (ES) cells (Fig. 1). Heterozygous *XPA* (*XPA*^{+/-}) mice (F₁) were obtained from crosses of chimaeric mice with C57BL/6 mice. From F₁ intercrosses the *XPA*^{-/-} offspring, however, were

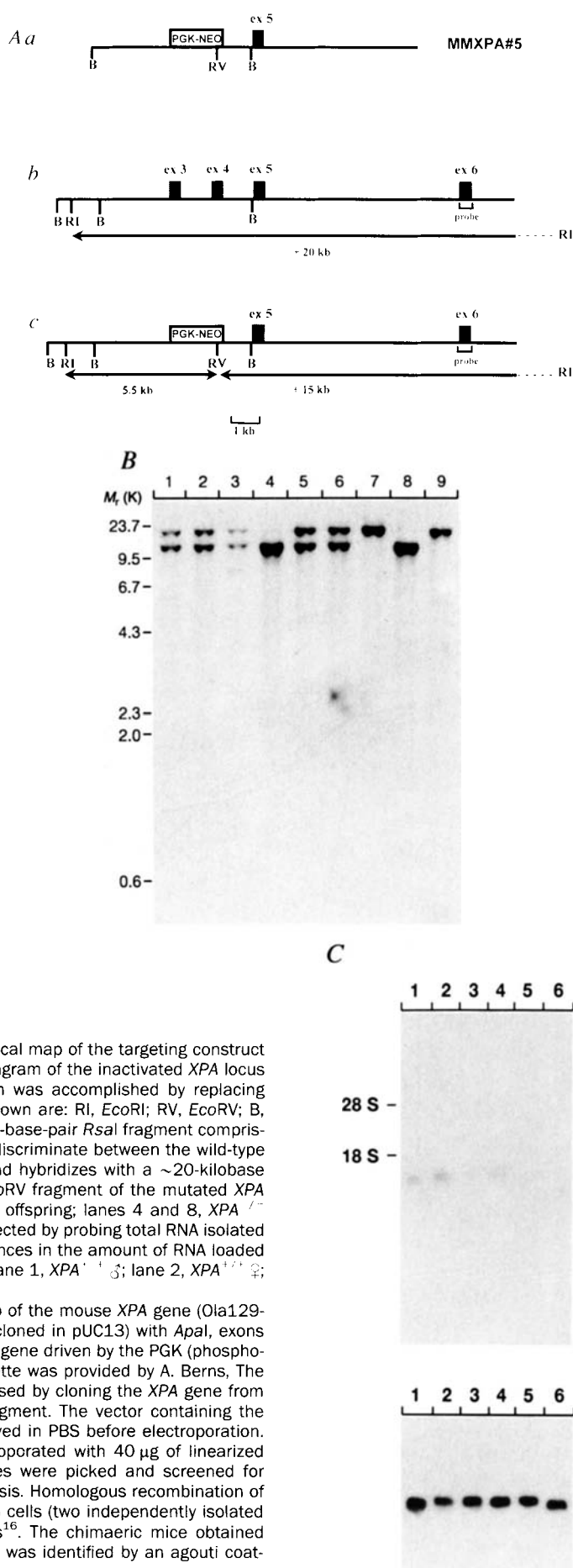


FIG. 1. Strategy of targeted inactivation of the mouse *XPA* gene. **Aa**, Physical map of the targeting construct MMXPA#5. **Ab**, Partial map of the endogenous mouse *XPA* locus. **Ac**, Diagram of the inactivated *XPA* locus after homologous recombination of the targeting construct. Inactivation was accomplished by replacing exons 3 and 4 by the *neo* marker cassette. Relevant restriction sites shown are: RI, *EcoRI*; RV, *EcoRV*; B, *BamHI*. **B**, Southern blot analysis of tail DNA (F₁ *XPA*^{+/-} intercross). A 650-base-pair *RsaI* fragment comprising exon 6 of the mouse *XPA* gene (see also **A**) was used as a probe to discriminate between the wild-type and mutated alleles. The probe is external to the targeting construct and hybridizes with a ~20-kilobase (kb) *EcoRI* fragment of the wild-type *XPA* locus and a ~15-kb *EcoRI/EcoRV* fragment of the mutated *XPA* locus. Lanes 7 and 9, *XPA*^{+/-} offspring; lanes 1, 2, 3, 5 and 6, *XPA*^{+/-} offspring; lanes 4 and 8, *XPA*^{-/-} offspring. **C**, Northern blot analysis. Upper panel, *XPA* transcripts were detected by probing total RNA isolated from mouse liver with the entire mouse *XPA* cDNA¹¹. Lower panel, differences in the amount of RNA loaded per lane were checked by rehybridizing the blot with a GAPDH probe¹². Lane 1, *XPA*^{+/-} ♂; lane 2, *XPA*^{+/-} ♀; lane 3, *XPA*^{+/-} ♂; lane 4, *XPA*^{+/-} ♀; lane 5, *XPA*^{-/-} ♂; lane 6, *XPA*^{-/-} ♀.

METHODS. The targeting construct MMXPA#5 (see **A**) comprises ~10 kb of the mouse *XPA* gene (Ola129-derived DNA). After digestion of a genomic 5.5-kb *BamHI* fragment (subcloned in pUC13) with *Apal*, exons 3 and 4 of the *XPA* gene were replaced by the neomycin-resistance (*neo*) gene driven by the PGK (phosphoglycerate kinase) promoter¹³ in a sense orientation. (The PGK-*neo* cassette was provided by A. Berns, The Netherlands Cancer Institute.) A 6-kb *BamHI* fragment (3' *BamHI* site raised by cloning the *XPA* gene from a genomic library) was inserted at the 3' part of the 5.5-kb *BamHI* fragment. The vector containing the targeting construct was made linear by digestion with *EcoRI* then dissolved in PBS before electroporation. ES cells (E14 (ref. 14), provided by A. Berns) were cultured and electroporated with 40 µg of linearized targeting vector as described^{14,15}. After 8 days, G418-resistant colonies were picked and screened for homologous recombination of the targeting vector by Southern blot analysis. Homologous recombination of the targeting construct occurred at a frequency of 2%. Gene-targeted ES cells (two independently isolated clones) were injected into C57BL/6 blastocysts by standard procedures¹⁶. The chimaeric mice obtained were mated with C57BL/6 mice. Transmission of E14-derived germ cells was identified by an agouti coat-colour in the offspring.

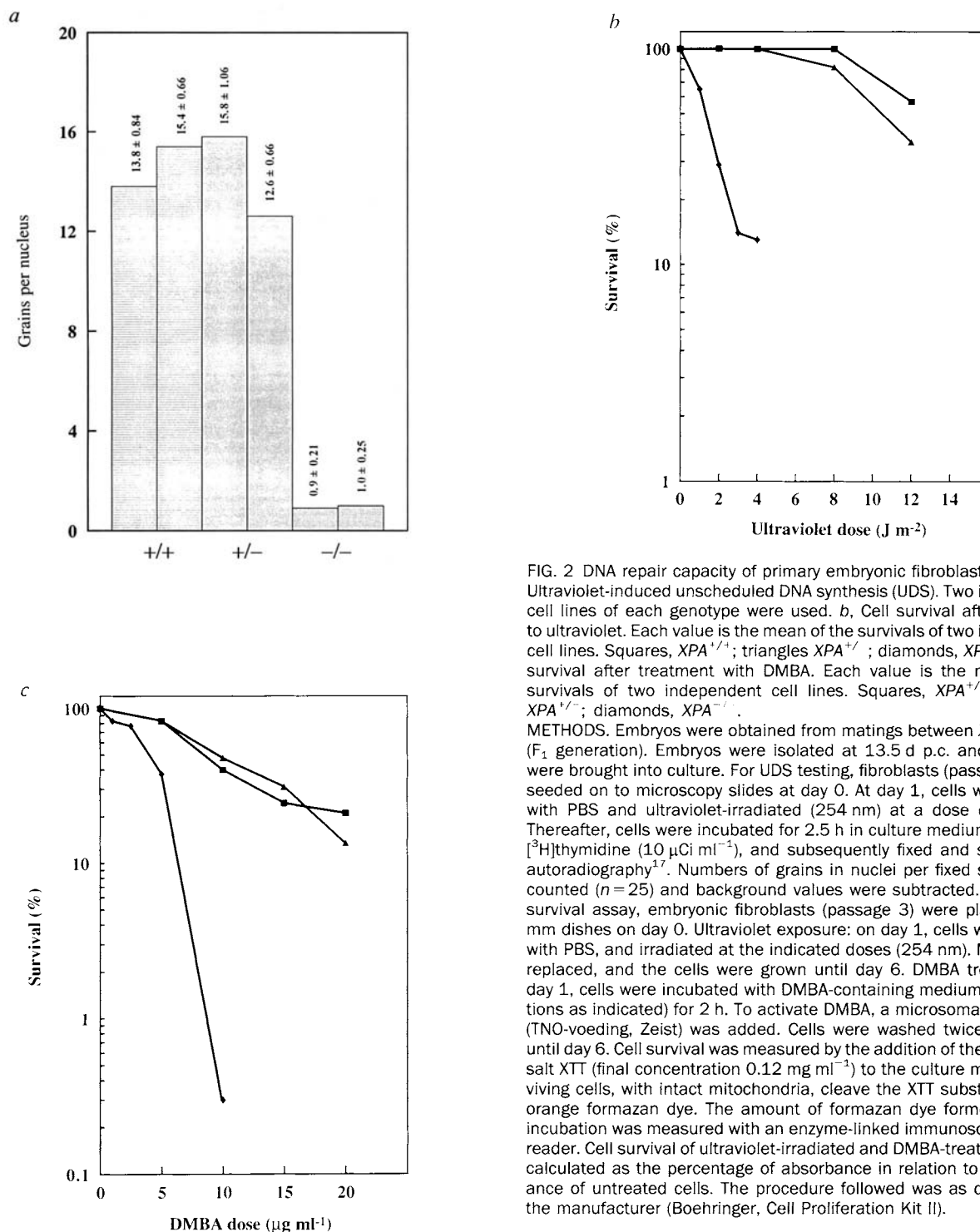


FIG. 2 DNA repair capacity of primary embryonic fibroblasts *in vitro*. **a**, Ultraviolet-induced unscheduled DNA synthesis (UDS). Two independent cell lines of each genotype were used. **b**, Cell survival after exposure to ultraviolet. Each value is the mean of the survivals of two independent cell lines. Squares, $XPA^{+/+}$; triangles, $XPA^{+/-}$; diamonds, $XPA^{-/-}$. **c**, Cell survival after treatment with DMBA. Each value is the mean of the survivals of two independent cell lines. Squares, $XPA^{+/+}$; triangles, $XPA^{+/-}$; diamonds, $XPA^{-/-}$.

METHODS. Embryos were obtained from matings between $XPA^{+/-}$ mice (F_1 generation). Embryos were isolated at 13.5 d p.c. and fibroblasts were brought into culture. For UDS testing, fibroblasts (passage 3) were seeded on to microscopy slides at day 0. At day 1, cells were washed with PBS and ultraviolet-irradiated (254 nm) at a dose of 16 J m^{-2} . Thereafter, cells were incubated for 2.5 h in culture medium containing [^3H]thymidine ($10 \mu\text{Ci ml}^{-1}$), and subsequently fixed and subjected to autoradiography¹⁷. Numbers of grains in nuclei per fixed square were counted ($n=25$) and background values were subtracted. For the cell survival assay, embryonic fibroblasts (passage 3) were plated on 15-mm dishes on day 0. Ultraviolet exposure: on day 1, cells were washed with PBS, and irradiated at the indicated doses (254 nm). Medium was replaced, and the cells were grown until day 6. DMBA treatment: on day 1, cells were incubated with DMBA-containing medium (concentrations as indicated) for 2 h. To activate DMBA, a microsomal S9-fraction (TNO-voeding, Zeist) was added. Cells were washed twice and grown until day 6. Cell survival was measured by the addition of the tetrazolium salt XTT (final concentration 0.12 mg ml^{-1}) to the culture medium. Surviving cells, with intact mitochondria, cleave the XTT substrate into an orange formazan dye. The amount of formazan dye formed after 2 h incubation was measured with an enzyme-linked immunosorbent assay reader. Cell survival of ultraviolet-irradiated and DMBA-treated cells was calculated as the percentage of absorbance in relation to the absorbance of untreated cells. The procedure followed was as described by the manufacturer (Boehringer, Cell Proliferation Kit II).

not obtained in the expected mendelian ratio. Of 732 pups born, 81 (11%) were of the $XPA^{-/-}$ genotype. To investigate the cause of the low yield of $XPA^{-/-}$ mice, embryos were isolated at defined embryonic stages. Up to 13 days post coitum (p.c.), the 3 genotypes were obtained at the expected ratio (that is, 11 $XPA^{+/+}$, 24 $XPA^{+/-}$ and 9 $XPA^{-/-}$). After day 13 p.c., abnormalities were observed in some $XPA^{-/-}$ embryos, namely growth retardation, a light appearance of the embryo in its intact yolk sac, and a decreased liver size. Histopathological examination of these embryos and their blood smears were indicative of anaemia and a disturbed liver haematopoiesis (results not shown). Apparently, ~50% of the $XPA^{-/-}$ embryos died of severe anaemia in the mid-fetal period.

Nevertheless, those $XPA^{-/-}$ mice that did come to term developed normally, at least until the age of 13 months. $XPA^{-/-}$ mice were healthy (no spontaneous tumour development) and fertile. No histological abnormalities were found in liver, blood, kidney, brain, heart, skin, spleen or lung. But we cannot exclude the possibility that some characteristic defects often found in xeroderma pigmentosum patients¹, for example neurological abnormalities, will develop later in the $XPA^{-/-}$ mice.

To ascertain whether the integration of the targeting construct has led to the inactivation of the XPA gene, total RNA from liver was analysed by northern blot. No stable (intact or truncated) XPA transcripts, nor any fusion product between XPA and *neo*, could be detected in the $XPA^{-/-}$ mice with the XPA mouse

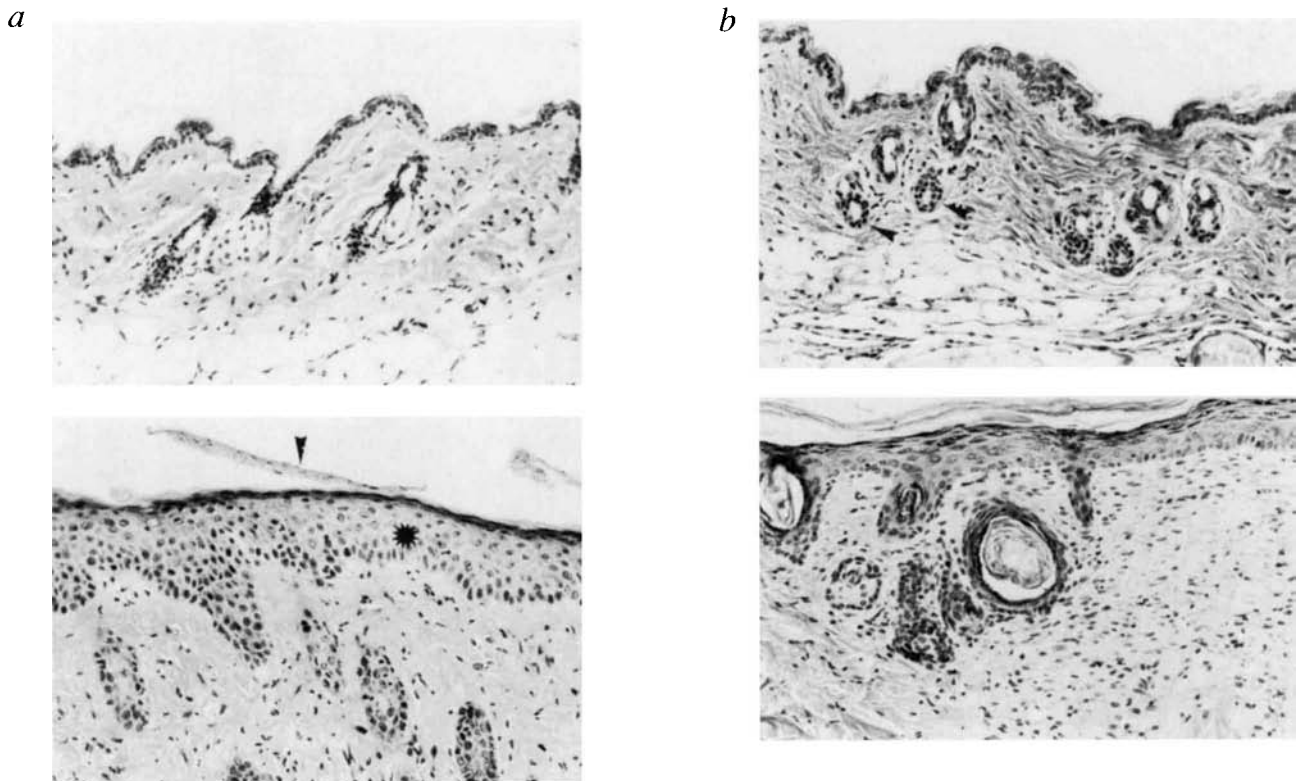


FIG. 3 Acute effects in the skin of $XPA^{-/-}$ mice after exposure to UV-B or treatment with DMBA. *a*, Histopathological examination of the acute effects of UV-B exposure on the skin of $XPA^{+/+}$ and $XPA^{-/-}$ mice. Upper panel, cross-section of $XPA^{+/+}$ skin; lower panel, cross-section of $XPA^{-/-}$ skin, showing epidermal hyperplasia and hyperkeratosis (indicated by an asterisk and an arrowhead, respectively). *b*, Histopathological examination of the effect of DMBA treatment on the skin of $XPA^{+/+}$ and $XPA^{-/-}$ mice. Upper panel, $XPA^{+/+}$ skin. Note the thin epidermis (1 or 2 cell layers) and normal hair shafts containing translucent hairs (arrowheads). Lower panel, $XPA^{-/-}$ skin, showing acanthosis (hyper-

regeneration), hyperkeratosis and abnormal hair structures, as well as hyperplastic regenerating hair shafts.

METHODS. Mice were shaved and irradiated with UV-B for 1 week (cumulative dose 1.2 kJ m^{-2} , American Philips F40 sunlamps, 250–400 nm) or treated with two applications of DMBA ($2 \times 10 \mu\text{g}$ dissolved in $100 \mu\text{l}$ acetone). Shortly after the final treatment (at day 7 in the UV-B experiment and at day 12 in the DMBA experiment) the animals were killed and part of the exposed skin was sampled and routinely processed for histopathology. Magnification, $\times 127$.

complementary DNA (Fig. 1C) and the *neo* coding sequence (results not shown) as a probe. In livers of $XPA^{+/+}$ mice the *XPA* mRNA content was approximately half of that in $XPA^{+/+}$ mice (Fig. 1C). This indicates that disruption of the *XPA* gene was effective.

Primary fibroblasts were isolated from embryos, and DNA repair activity of the cells was measured by ultraviolet-induced unscheduled DNA synthesis (UDS) and cell survival after exposure to ultraviolet or to the genotoxic carcinogen DMBA. DNA repair activity, as determined by UDS, is hardly detectable in the $XPA^{-/-}$ fibroblasts (see Fig. 2a). Survival of $XPA^{-/-}$ fibroblasts is extremely low after exposure to ultraviolet (Fig. 2b) or treatment with DMBA (Fig. 2c). At an ultraviolet dose of 4 J m^{-2} , the $XPA^{-/-}$ fibroblasts showed a survival of $\sim 10\%$, compared with a virtual 100% survival of $XPA^{+/+}$ and $XPA^{+/+}$ fibroblasts. At a dose of $10 \mu\text{g ml}^{-1}$ DMBA (in culture medium), $XPA^{-/-}$ fibroblasts showed a survival of $\sim 0.3\%$, compared with the $\sim 45\%$ survival of $XPA^{+/+}$ and $XPA^{+/+}$ fibroblasts. This increased sensitivity to genotoxic agents is comparable to that observed in cells isolated from xeroderma pigmentosum patients^{8,9}. In both *in vitro* assays, $XPA^{+/+}$ fibroblasts behaved as wild-type cells, which argues against a dominant-negative effect of the mutation and a gene dose effect.

To establish whether the $XPA^{-/-}$ mice are susceptible to developing skin cancer, they were exposed to (1) low daily UV-B doses¹⁰, gradually increasing to $\sim 310 \text{ J m}^{-2}$ or to (2) low doses of DMBA ($10 \mu\text{g}$ once weekly, total 8 applications). Within one week, $XPA^{-/-}$ mice showed hyperkeratosis and erythema on the

ultraviolet-exposed dorsal skin, and massive epidermal hyperplasia was observed (Fig. 3a). After two DMBA treatments, $XPA^{-/-}$ mice showed extensive hair loss and crust formation at the application site. Marked changes such as necrosis of the epidermis, including appendages (hair follicles and sebaceous glands), as well as several features indicative of regeneration of epithelia and dermal fibroblasts, were observed (e.g. 3b). In the skin of ultraviolet-irradiated or DMBA-treated $XPA^{+/+}$ and $XPA^{+/+}$ mice, these acute effects were absent. Thus the loss of a functional NER pathway seems to render the skin of the $XPA^{-/-}$ mice very sensitive to genotoxic agents. After 6 weeks of UV-B irradiation, the $XPA^{-/-}$ mice started to develop abnormalities of the eye (not found in $XPA^{+/+}$ and $XPA^{+/+}$ mice). Initially corneal opacity was observed and, as irradiation proceeded, some of the $XPA^{-/-}$ mice developed Bowenoid lesions (results not shown). Owing to the eye lesions, occurring with varying severity in almost all $XPA^{-/-}$ mice, the UV-B exposure was discontinued after 14 weeks (accumulated dose of $\sim 22 \text{ kJ m}^{-2}$). Shortly thereafter the first skin tumours appeared and 75% (15 out of 20) of the $XPA^{-/-}$ mice had developed one or more skin tumours at 25 weeks (see Fig. 4a). Tumours were present only on UV-B exposed areas, and were squamous cell carcinomas (Fig. 4c) or precursor lesions, solar keratoses. $XPA^{+/+}$ and $XPA^{+/+}$ mice did not develop tumours within the same observation period (Fig. 4a). After 6 weeks of DMBA treatment, papillomas became visible on the skin of $XPA^{-/-}$ mice. Within 19 weeks, 59% of the $XPA^{-/-}$ mice (10/17) carried one or more papillomas (Fig. 4b, d). For $XPA^{+/+}$ and

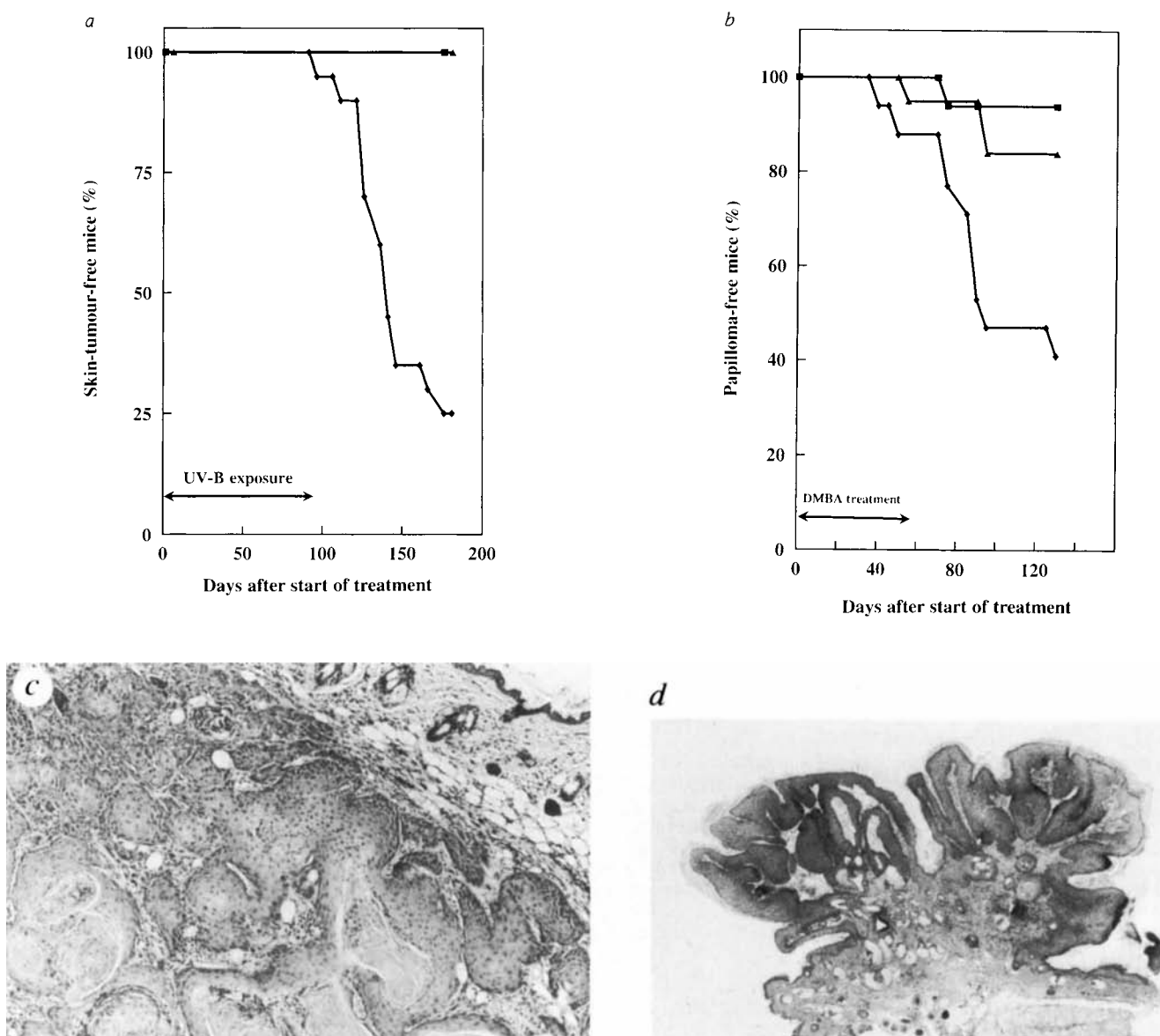


FIG. 4 Tumour development on the skin of UV-B-exposed and DMBA-treated mice. *a*, Incidence and latency time of tumours arising on UV-B-treated skins of mice of the three genotypes. Squares, $XPA^{+/+}$ mice; triangles, $XPA^{+/-}$ mice; diamonds, $XPA^{-/-}$ mice. *b*, Incidence and latency time of papillomas on DMBA-treated skins of mice of the three genotypes. Squares, $XPA^{+/+}$ mice; triangles, $XPA^{+/-}$ mice; diamonds, $XPA^{-/-}$ mice. *c*, Histopathological examination of UV-B-induced skin tumours of $XPA^{-/-}$ mice. The figure shows a typical example of a squamous cell carcinoma, predominantly found in these mice. Magnification, $\times 69$. *d*, Histopathological examination of DMBA-induced skin tumours of $XPA^{-/-}$ mice. The figure shows a typical example of a papilloma, the only tumour type found in this experiment. Magnification, $\times 20$.

METHODS. For the short-term UV-B experiment, $XPA^{+/+}$ ($n=7$), $XPA^{+/-}$ ($n=13$) and $XPA^{-/-}$ mice ($n=20$) were shaved once a week and

exposed to short (between 25 and 60 min), daily irradiations with UV-B, initially 130 J m^{-2} and gradually increasing to 310 J m^{-2} within 9 weeks (American Philips F40 sunlamps, 250–400 nm). After 14 weeks the UV-B treatment was stopped. Untreated $XPA^{+/+}$ ($n=3$), $XPA^{+/-}$ ($n=6$) and $XPA^{-/-}$ mice ($n=4$) were only shaved weekly. All mice were checked twice a week for the development of skin tumours. Tumour-bearing animals were killed, tumours were isolated and routinely processed for histopathology. For the short-term DMBA experiment, $XPA^{+/+}$ ($n=18$), $XPA^{+/-}$ ($n=19$) and $XPA^{-/-}$ mice ($n=17$) were treated once a week on the shaved back with $10 \mu\text{g}$ DMBA dissolved in $100 \mu\text{l}$ acetone. After 8 applications the DMBA treatment was stopped. Control $XPA^{-/-}$ mice ($n=6$) were shaved weekly and received only the solvent (acetone). All mice were checked twice a week for the development of papillomas. Tumour-bearing animals were killed, tumours were isolated and routinely processed for histopathology.

$XPA^{+/+}$ mice these numbers were 3/19 and 1/18, respectively (Fig. 4b). No progression of DMBA-induced papilloma to carcinoma was observed, not even after an observation period of 40 weeks.

From the lack of capacity to repair DNA damage and the highly increased sensitivity to genotoxic agents resulting in an increased incidence of tumours, we conclude that our $XPA^{-/-}$ mice strongly mimic the phenotype of xeroderma pigmentosum

patients. We therefore believe that this mouse model is useful to explain the function of the XPA protein in NER, and the role of NER in the process of spontaneous or induced carcinogenesis. Moreover, our data also indicate that the XPA -deficient mice might be useful for sensitive short-term *in vivo* carcinogenicity testing of genotoxic agents. The preliminary results from continuing experiments with benzo[a]pyrene (oral administration) seem to support this. □

Received 13 June; accepted 24 July 1995.

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ACKNOWLEDGEMENTS. We thank B. van Middelaar, H. Näring, T. Hesp and H. Sturkenboom for their biotechnical support; J. de Wit and G. Weeda for their assistance in UDS testing; J. Toonstra for the analysis of UV-B-induced skin tumours, A. Piersma for his help with the analysis of embryos, G. Mohn for critical reading of the manuscript and K. Post for general assistance. This work was supported in part by a grant under the Commission of the European Communities Biotechnology Programme.

Role of IRS-2 in insulin and cytokine signalling

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THE protein IRS-1 acts as an interface between signalling proteins with Src-homology-2 domains (SH2 proteins) and the receptors for insulin, IGF-1, growth hormone, several interleukins (IL-4, IL-9, IL-13) and other cytokines^{1–7}. It regulates gene expression and stimulates mitogenesis, and appears to mediate insulin/IGF-1-stimulated glucose transport⁸. Thus, survival of the IRS-1^{-/-} mouse with only mild resistance to insulin was surprising^{9,10}. This dilemma is provisionally resolved with our discovery of a second IRS-signalling protein. We purified and cloned a likely candidate called 4PS from myeloid progenitor cells and, because of its

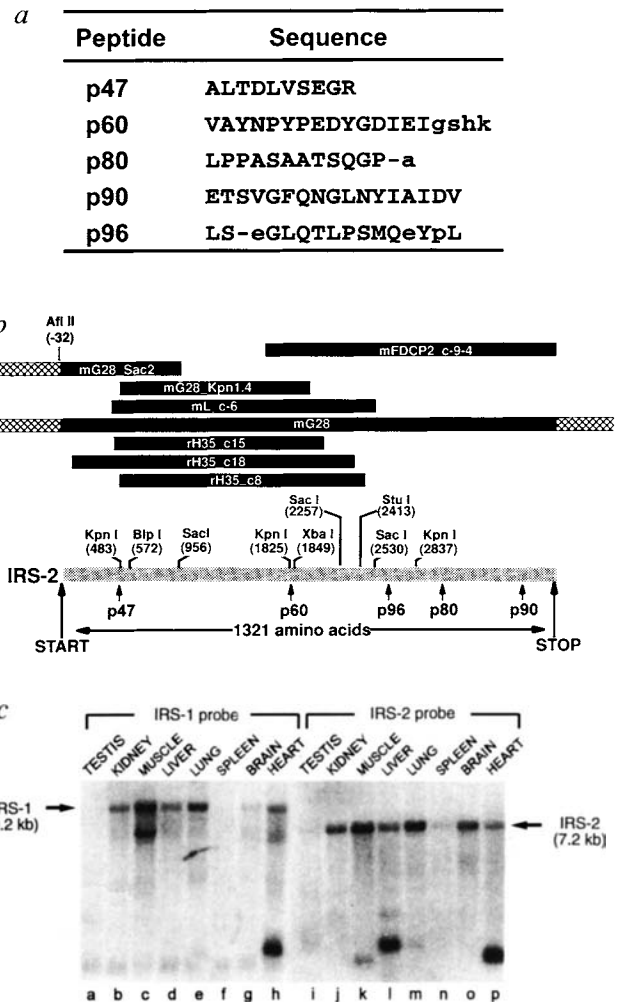


FIG. 1 The molecular cloning of IRS-2. **a**, The amino-acid sequences of five unique tryptic peptides obtained from 4PS are shown in upper case for full confidence, in lower case for less confidence. Dashes indicate positions that could not be assigned. **b**, Map of overlapping cDNA and genomic DNA clones encoding mouse and rat IRS-2. Mouse cDNA (mFDCP2c-9-4) was initially isolated from a cDNA library prepared with mRNA from FDC-P2 cells screened with an oligonucleotide based on the amino-acid sequence of p60. **c**, Northern blot of mouse tissue (Clontech) was hybridized at high stringency with an IRS-2 (2,987–3,325 bp) (lanes i–p) or IRS-1 cDNA probe (2,834–3,136 bp) (lanes a–h). The ³²P-labelled cDNA probes were prepared with Multiprime DNA Labeling System (Amersham)¹⁴.

METHODS. Cell lysates from insulin-stimulated FDC-P2 cells (20 × 10⁹) were clarified by centrifugation and passed over the Sepharose-immobilized GST-fusion protein containing the N-terminal SH2 domain from anti-p85 antibody, washed, and eluted with 100 mM Tris-HCl

(pH 7.4) containing 40 mM glutathione, 40 mM DTT, 250 mM NaCl, 0.2 mM sodium orthovanadate and 0.4 mM PMSF. The concentrated eluate was resolved by 7.5% SDS-PAGE, transferred to PVDF membranes and stained with 0.5% Ponceau-S. The area corresponding to 4PS was excised and digested *in situ* with trypsin as described³¹. The eluted peptides were separated by narrow-bore high-performance liquid chromatography using a Vydac C18 reverse-phase column (2.1 mm × 150 mm). The sequences of five unique tryptic peptides (p47, p60, p80, p90, p96) were determined by automated Edman degradation as described^{32,33}. The optimized oligonucleotide probe based on the sequence of p60 (**a**) was prepared for library screening with a pair of oligonucleotides containing a 10-nucleotide overlap (underlined sequence), as previously described: 5'-GTGGCCCTACAACCCATACCTGAGGAC-3' and 5'-AATCTCAATGTCGCCATAGTCCTCAGGG-3' (ref. 14). About 10⁶ plaques were screened with the ³²P-labelled oligonucleotide probe (2.5 × 10⁶ c.p.m. ml⁻¹) and one cDNA clone (mFDCP2c-9-4) was obtained from the mouse FDC-P2 cell poly(T)-primed cDNA library prepared in λExlox (Novagen), and one genomic clone (mG28) was isolated from a mouse genomic library in λFIX (Stratagene). Both strands of the 2.4-kb cDNA insert were sequenced with Sequenase (US Biochem.) using specific primers selected at convenient intervals. A mouse lung cDNA library was screened with the 5' end of clone mFDCP2c-9-4 revealing a new cDNA (mLc-6) that partially overlapped with mFDCP2c-9-4. Two genomic fragments obtained by digestion of mG28 with KpnI (mG28Kpn1.4) or SacI (mG28Sac2) which hybridized with the 5' end of mLc-6, were subcloned and sequenced. The sequences were aligned and the open reading frame of 3,963 nucleotides was identified using the EUGENE and SAM programs. Rat cDNA corresponding to the 5' end of mouse IRS-2 was identified with mG28Sac2 in a rat H35 hepatoma cDNA library (rH35c15, rH35c18, and rH35c8). Most of the mouse genomic 5' sequence was confirmed by alignment with rH35c18, although cDNA clones encoding the extreme 5' end were never obtained. Northern blots were hybridized overnight at 42 °C with specific probes as described¹⁴.

resemblance to IRS-1, we designate it IRS-2. Alignment of the sequences of IRS-2 and IRS-1 revealed a highly conserved amino terminus containing a pleckstrin-homology domain and a phosphotyrosine-binding domain, and a poorly conserved carboxy terminus containing several tyrosine phosphorylation motifs. IRS-2 is expressed in many cells, including tissues from IRS-1^{-/-} mice¹¹, and may be essential for signalling by several receptor systems.

Using a Sepharose-coupled SH2 domain derived from p85, about 5 µg 4PS were partially purified from 20 × 10⁹ insulin-stimulated FDC-P2 cells⁴. Five tryptic peptides derived from 4PS were subjected to automated Edman microsequencing: two (p60 and p90) were ~35% identical to IRS-1, whereas the other three (p47, p80 and p96) had no significant homology with any GeneBank entries (Fig. 1a).

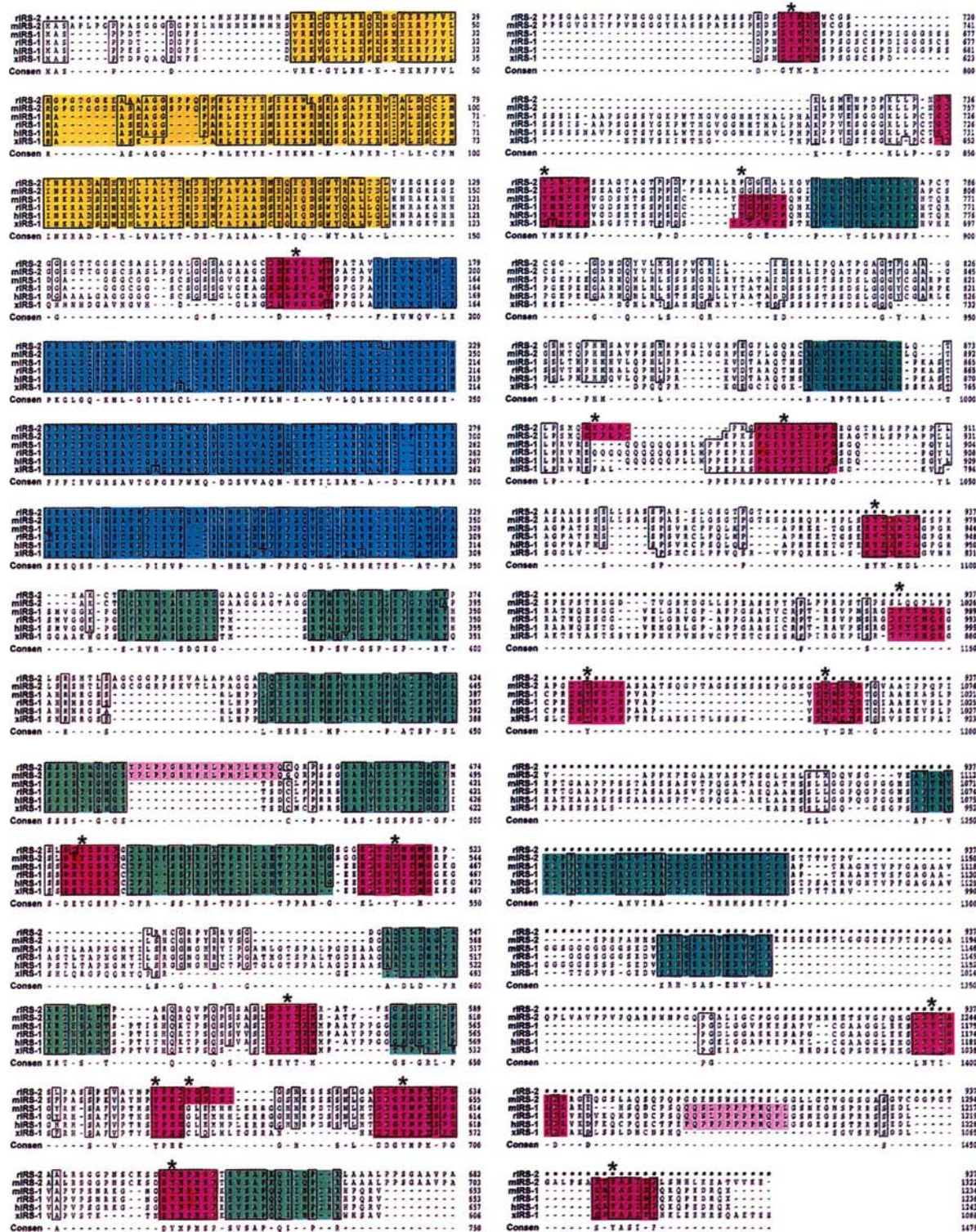


FIG. 2 Sequence alignment between IRS-1 and IRS-2. An optimized alignment of the complete mouse (m) and partial rat (r) amino-acid sequences of IRS-2 against the complete mouse (m), rat (r), human (h) and *Xenopus* (x) IRS-1 sequences was obtained using the PILEUP program (Genetic Computer Group, Madison, Wisconsin). Gaps are indicated by dashed lines, identities are boxed and the consensus sequence is shown. The IH-1^{PH} and the

IH-2^{PTB} domains are shown in yellow and blue, respectively; conserved tyrosine-phosphorylation motifs are pink, and the putative phosphorylation site is labelled with an asterisk. Conserved regions of unknown function in the C terminus are shown in green and proline-rich motifs in purple. Incomplete IRS-2 sequence is indicated by small square dots.

A complementary DNA library from the FDC-P2 cells was screened with an optimized nucleotide probe based on the amino-acid sequence of p60 (Fig. 1a). A single clone was isolated (mFDCP2c-9-4) which contained a 2.4-kilobase (kb) insert that was 41% identical to nucleotides 1,353–3,538 of mouse IRS-1 (Fig. 1b). The conceptual translation was 35% identical to amino-acid residues 451–1,234 in mouse IRS-1, and it contained the exact amino-acid sequences of p60, p80, p90 and p96. Based on these observations, we designated the putative protein 'IRS-2'.

The complete coding region of IRS-2 (3,963 nucleotides) was assembled from overlapping mouse cDNA and genomic fragments, and confirmed with homologous rat clones (Fig. 1b). Northern analysis indicated that the principal messenger RNA transcript in mice for IRS-2 is 7.2 kb, whereas the transcript for IRS-1 is 9.2 kb (Fig. 1c). IRS-2 is not limited to haematopoietic cells, as it is expressed in skeletal muscle, lung, brain, liver, kidney, heart and spleen (Fig. 1c). Moreover, it was detected with a similar tissue distribution in IRS-1^{-/-} mice, suggesting that it is the alternative insulin receptor substrate in that background¹¹.

The IRS-2 cDNA encodes a peptide of relative molecular mass 145K, which is 10K longer than IRS-1. Alignment of murine

IRS-2 with IRS-1 from *Xenopus*¹², mouse¹³, rat¹⁴ and human¹⁵ sources revealed two IRS-homology (IH-1 and IH-2) domains at the amino terminus (Fig. 2). The IH-1 domain contains 101 (IRS-1) to 112 (IRS-2) amino acids with 69% identity, whereas the IH-2 domain contains 156 (IRS-1) to 160 (IRS-2) amino acids with 75% identity (Fig. 2). By contrast, the C-terminal regions of IRS-1 and IRS-2 are 35% identical; however, approximately 20 common or unique tyrosine-phosphorylation motifs are located in relatively similar positions, suggesting that both IRS-1 and IRS-2 bind multiple SH-2 proteins, including p85, Grb-2 and SH-PTP2 (Fig. 2).

The expression and tyrosine phosphorylation of endogenous IRS-2 was investigated in ordinary FDC-P2 cells, and compared to recombinant IRS-1 or IRS-2 expressed in 32D cells which do not contain these endogenous proteins⁵. Cell extracts were immunoprecipitated with antibodies against PY, IRS-2 or IRS-1, and tyrosine phosphorylation was detected by immunoblotting with anti-PY antibody. During insulin or IL-4 stimulation, anti-PY and anti-IRS-2 immunoprecipitated tyrosine-phosphorylated IRS-2 from FDC-P2 cells and 32D^{IRS2} cells (Fig. 3a, b), whereas anti-IRS-1 did not (Fig. 3c). In contrast, anti-PY and anti-IRS-1 strongly immunoprecipitated IRS-1 from 32D^{IRS1} cells (Fig. 3a, c), whereas anti-IRS-2 did not crossreact

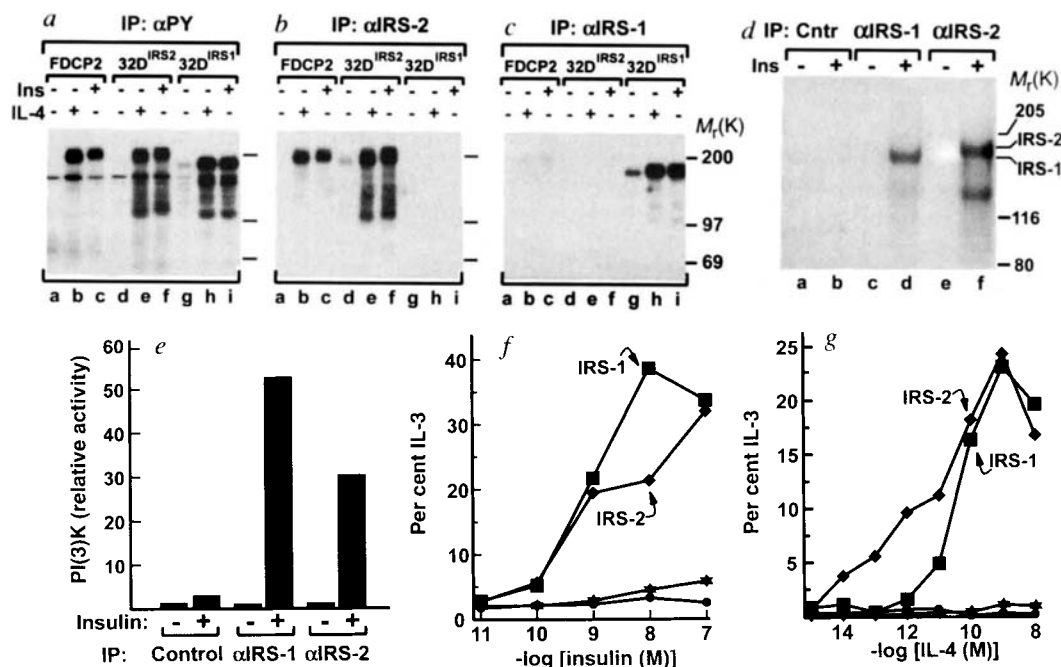
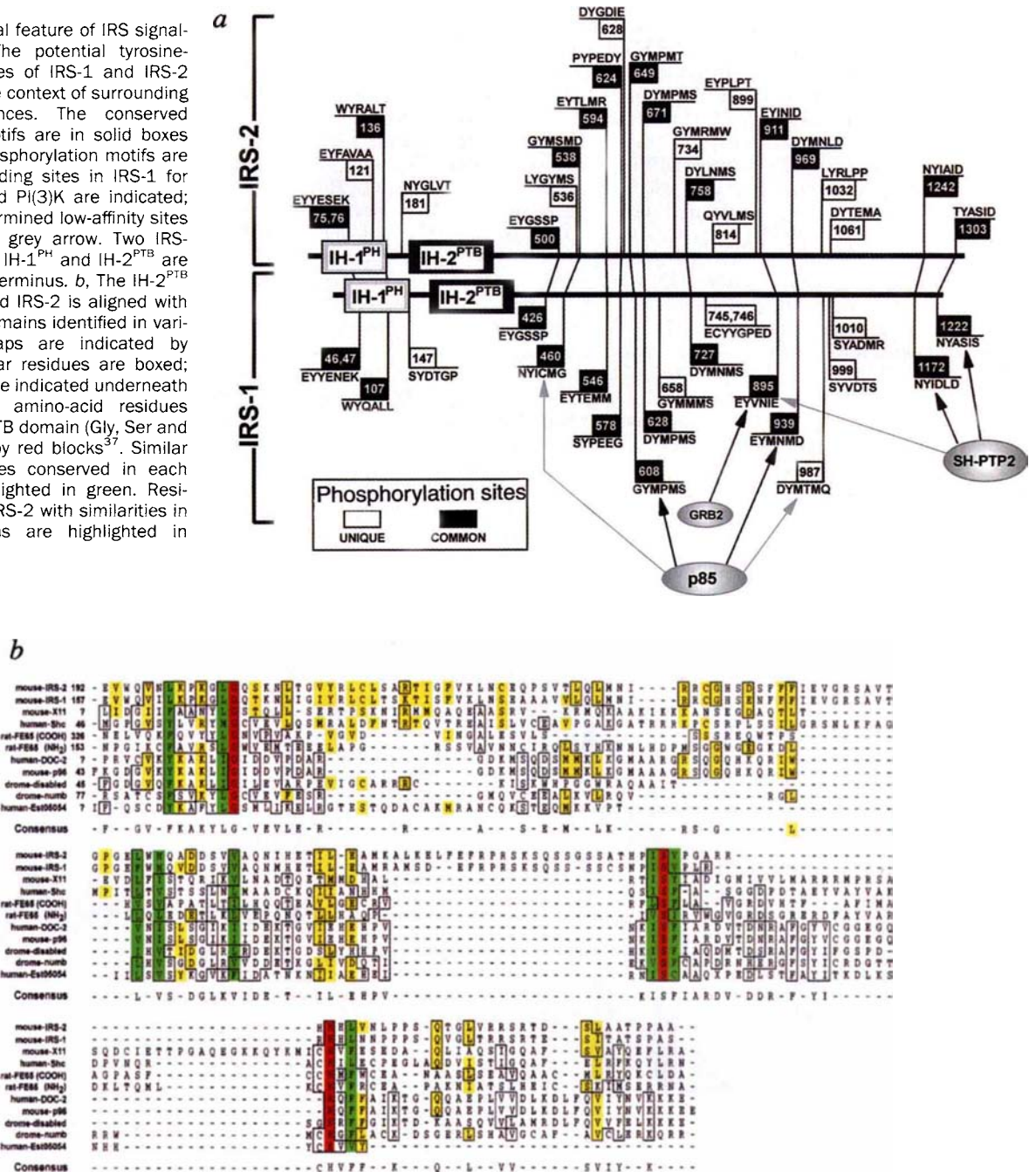


FIG. 3 The expression and function of IRS-2 in FDC-P2 cells, 32D cells and Fao hepatoma cells. a–c, Quiescent FDC-P2 cells, or 32D cells expressing the receptors for insulin and IL-4 (32D^{IR/IL4R}) and either IRS-1 or IRS-2 were incubated without or with 100 nM insulin or 10 nM IL-4 for 1 min. Cell lysates were immunoprecipitated with antibodies against PY, IRS-2 or IRS-1 (αPY, αIRS-2, αIRS-1), separated on 7.5% SDS-PAGE, and immunoblotted with αPY as described³⁴. Quiescent rat Fao hepatoma cells (d) were incubated without or with 100 nM insulin for 1 min and immunoprecipitated with non-immune serum, αIRS-1 or αIRS-2, and immunoblotted with αPY; e, Phosphatidylinositol-3-OH kinase activity associated with the immunocomplexes was determined³⁴. f and g, insulin and IL-4 stimulated [³H]thymidine incorporation into 32D cells (circles) or 32D^{IR/IL4R} cells before (stars) or after expression of IRS-1 or IRS-2 (as indicated).

METHODS. Antibodies against IRS-1 and IRS-2 were prepared in rabbits (HRP Corp.) as described¹⁴. αIRS-2 was obtained as a GST-fusion protein containing residues 619–746 of mouse IRS-2 (ref. 20). αIRS-1 (residues 1,221–1,234 of rat IRS-1) were made with synthetic peptides coupled to keyhole limpet haemocyanin³⁵; αPY antibodies were rabbit polyclonal³⁶ or mouse monoclonal 4G10 (UBI). An IRS-2 expression vector was assembled in pCMV^{his} from two partial cDNA inserts (mFDCP2c-9-4 and mLc-6) and a genomic fragment (mG28Sac2) by

using *NotI*, *BlnI* and *AflII* restriction sites, and blunt-ended *SpeI* and *HindIII* subcloning sites. The correct orientation and reading frame was confirmed by DNA sequencing. 32D cells or cells expressing the IR and IL4R (32D^{IR/IL4R}) were transfected stably with IRS-1 or IRS-2 cDNA by electroporation as described¹⁸. Lysates from quiescent 32D cells incubated for 1 min in the absence or presence of 100 nM insulin or 10 nM IL-4 were immunoprecipitated with the indicated antibodies, separated by 7.5% SDS-PAGE and immunoblotted with αPY³⁴. Fao cells were grown to confluence in RPMI-1640 medium and incubated with insulin (100 nM) for 10 min, and immune complexes were immunoblotted with αPY or assayed for PI(3)K activity as described¹⁶. Incorporation of [³H]thymidine into 32D cells was used to quantify factor-induced proliferation. 32D cells (2 × 10⁵ cells per ml) were incubated for 36 h in RPMI-1640 medium containing 10% FBS and the indicated growth factors or 5% WEHI-3-conditioned medium, incubated for 3 h with [³H]thymidine and collected on glass filters (Whatman)⁵. Radioactivity was quantified by scintillation counting in ACS scintillation cocktail (Amersham). All determinations were carried out in triplicate, and data are reported as the percentage of incorporation of radioactivity into cells growing in medium supplemented with 5% WEHI-3-conditioned medium¹⁸.

FIG. 4 The structural feature of IRS signaling proteins. **a**, The potential tyrosine-phosphorylation sites of IRS-1 and IRS-2 are presented in the context of surrounding amino-acid sequences. The conserved phosphorylation motifs are in solid boxes and the unique phosphorylation motifs are in open boxes. Binding sites in IRS-1 for GRB2, SH-PTP2 and PI(3)K are indicated; experimentally determined low-affinity sites are indicated by a grey arrow. Two IRS-homology domains, IH-1^{PH} and IH-2^{PTB} are indicated at the N terminus. **b**, The IH-2^{PTB} domain in IRS-1 and IRS-2 is aligned with the putative PTB domains identified in various proteins³⁷. Gaps are indicated by dashed lines, similar residues are boxed; identical residues are indicated underneath (consensus). Three amino-acid residues diagnostic for the PTB domain (Gly, Ser and His) are indicated by red blocks³⁷. Similar hydrophobic residues conserved in each sequence are highlighted in green. Residues in IRS-1 and IRS-2 with similarities in other PTB domains are highlighted in yellow.



(Fig. 3b). The co-migration during SDS-PAGE of endogenous and recombinant IRS-2 slightly above IRS-1 suggested that we had the full-length cDNA encoding IRS-2.

The common ability of tyrosine-phosphorylated IRS-1 and IRS-2 to engage phosphatidylinositol-3-OH kinase (PI(3)K) was investigated in rat Fao hepatoma cells, which contain IRS-1 and a slower-migrating protein called pp185^{HMW} (ref. 16). Both IRS-1 and IRS-2 were immunoprecipitated from Fao hepatoma cells and immunoblotted with anti-PY (Fig. 3d); pp185^{HMW} appears to be identical to IRS-2. Insulin stimulated tyrosine phosphorylation of both IRS-1 and IRS-2 in Fao cells, and as expected, IRS-2 migrated slightly above IRS-1 (Fig. 3d). Interestingly, anti-IRS-2 coprecipitated a 125K tyrosine-phosphorylated protein which was not found in the anti-IRS-1 immunocomplex (Fig. 3d, lane f). Phosphatidylinositol-3-OH kinase associated with both IRS-1 and IRS-2 following insulin stimulation (Fig. 3e), suggesting that both IRS-proteins serve as interfaces between the insulin receptor and PI(3)K in the same cell background.

As untransfected 32D cells do not contain IRS-1 or IRS-2, many biological responses, including the activation of PI(3)K and p70^{s6k} and mitogenesis, are insensitive to insulin/IGF-1, IL-4, IL-13 and other cytokines^{5,7,17,19}. Moreover, overexpression of the insulin receptor or the IL-4 receptor in 32D cells does not rescue the mitogenic response (Fig. 3f, g). However, expression of either IRS-2 or IRS-1 together with these receptors rescued insulin and IL-4 stimulated DNA synthesis (Fig. 3f, g). Thus IRS-1 and IRS-2 are interchangeable as mediators of insulin- and IL-4-stimulated mitogenesis in 32D cells. Interestingly, IRS-2 coupled more sensitively to the IL-4 receptor system than IRS-1.

Among the eight identified tyrosine-phosphorylation motifs in IRS-1, six are well conserved in IRS-2 (tyrosines at positions 649, 671, 911, 969, 1,242 and 1,303). The SH2 domains in p85 bind strongly to Y₆₀₈MPM and Y₉₃₉MNM in IRS-1 (ref. 20), which correspond to Y₆₄₉MPM and Y₉₆₉MNL in IRS-2 (Fig. 4a); however, the substitution of Met 972 by Leu may alter the interaction. Nevertheless, there are eight other YXXM motifs in

IRS-2 which may bind p85 after tyrosine phosphorylation (Fig. 4a). The SH2 domain of Grb-2 binds to a Y₈₉₅VNI motif in IRS-1 (refs 17, 20), which corresponds to Y₉₁₁INI in IRS-2 (Fig. 4a). IRS-1 also binds to SH-PTP2 at the Y₁₁₇₂IDL and the Y₁₂₂₂ASI motifs^{20, 22}, which corresponds to the Y₁₂₄₂IAI and Y₁₃₀₃ASI motifs in IRS-2; however, the spacing between these motifs is 10 residues longer in IRS-2, which may alter the regulation of SH-PTP2 (Fig. 4a). Several other potential tyrosine-phosphorylation motifs unique to IRS-1 or IRS-2 may play distinct roles in signalling (Fig. 4a). Moreover, the C terminus possesses several other conserved motifs in an otherwise dissimilar peptide backbone, which could mediate protein-protein interactions (Fig. 2).

The IH-1 domain in IRS-1 was previously recognized as a pleckstrin-homology (PH) domain^{23, 24}, and is best designated as IH-1^{PH} to emphasize this fact (Figs 2, 4a). The high degree of identity between the IH-1^{PH} domains in IRS-1/IRS-2 suggests a specific function for this region. Deletion of the IH-1^{PH} domain from IRS-1 significantly impairs coupling to an ordinary level of insulin receptors²⁵. However, the IH-1^{PH} domain is not essential for IRS-1 phosphorylation, because a high level of insulin receptors in 32D cells rescues tyrosine phosphorylation of IRS-1 molecules lacking this domain²⁵.

Insulin-stimulated tyrosine phosphorylation of IRS-1 and Shc is mediated by an NPXY motif in the juxtamembrane region of the insulin receptor β -subunit^{26, 27}; a similar motif exists in the

IL-4 receptor and is required for IRS phosphorylation²⁸. A phosphotyrosine-binding (PTB) domain that recognizes phosphorylated NPXY motifs has been identified in Shc and other proteins^{29, 30}. Alignment of the IH-2 domain in IRS-1 and IRS-2 with the PTB domains revealed the presence of three conserved amino acids (Gly 204, Ser 314, and His 322 in IRS-2) surrounded by similar hydrophobic residues which are diagnostic for the PTB domain (Fig. 4b). We propose the designation, IH-2^{PTB}, to emphasize this similarity. Moreover, a fusion protein with glutathione-S-transferase that contained the IH-2^{PTB} domain of IRS-1 or IRS-2 bound specifically to the insulin-receptor-derived sequence, LYASS-NPE[pY₉₆₀]-LSASDV (data not shown). Moreover, deletion of the IH-2^{PTB} domain impairs IRS-1 phosphorylation, suggesting that this region is important for receptor coupling.

The identification of IRS-2 provides new insight into the modular structure and function of the IRS signalling proteins. Presumably, the IRS signalling system provides a means for signal amplification by eliminating the stoichiometric constraints encountered by receptors that directly recruit SH2 proteins to their autophosphorylation sites. Moreover, IRS proteins dissociate the intracellular itinerary of the signalling complex from the endocytic pathways ordinarily followed by the activated receptor. The shared use of IRS proteins by multiple receptors is likely to reveal important connections between various hormones and cytokines that were previously unrecognized, or observed but unexplained. □

Received 10 April; accepted 5 July 1995.

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ACKNOWLEDGEMENTS. We thank M. E. Patti, S. Pons and C. R. Kahn for discussion and for sharing unpublished data, and R. Robinson, V. Bailey, J. Neveu and D. Lizotte for help with peptide isolation and microsequencing. This work was supported by grants from the NIH to M.F.W. X.J.S. is a fellow of the Juvenile Diabetes Foundation and M.G.M. was partially supported by the Albert J. Ryan Foundation at Harvard Medical School.

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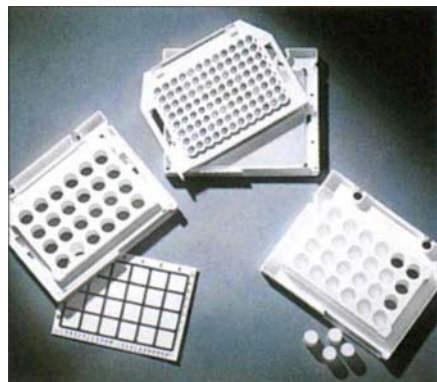
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Annual checkup

Browse some of the items that may be on display at the annual research meeting of the National Institutes of Health — new information for yeast transformation, mathematical software, immunological reagents and more.

WALLAC has developed three **cassettes** that expand the range of MicroBeta Plus, a multi-detector instrument that counts beta, gamma and luminescent labels (*Reader Service No. 100*). The instrument measures the results of assays directly in the formats in which the



New cassettes for the MicroBeta Plus.

tests were performed: microplates, filterplates, filtermats and tubes. The new cassettes are reusable accessories that allow the user to perform an even wider variety of advanced assays. Designed for Cherenkov counting, one cassette integrates a 96-well filtermat, MeltiLex scintillator and a stainless steel insert to prevent crosstalk and eliminate liquid waste. Another holds a 24-well filtermat and is should prove useful for receptor binding and tissue culture studies. The third is a 24-well cassette designed to hold Beckman Redi-Caps that scintillate as a solid with yttrium silicate, eliminating cocktail and liquid waste.

Beckman has announced **on-line information** access on the World Wide Web (*Reader Service No. 101*). Now access to the latest advancements in protein and DNA research via application notes, technical

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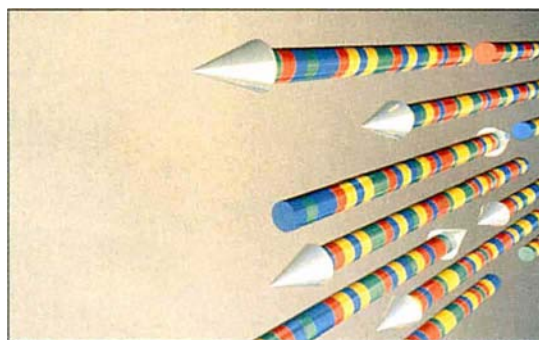
Zymo Research has developed a new kit for **yeast transformation** called the Frozen-EZ yeast transformation kit (*Reader Service No. 102*). This method is said to prepare competent yeast cells in as little as ten minutes that can be used directly for transformations or stored below -70°C for future use (storage of competent cells below -70°C is said to increase the transformation efficiency). The manufacturer states that freshly prepared or frozen competent cells can be transformed with plasmid DNA in 45 min using a one-solution, one-step protocol. Transformation efficiency for *Saccharomyces cerevisiae* is stated to be about 10^5 transformants per microgram of plasmid DNA. This kit is also suitable for transformation of *Pichia pastoris*, *S. pombe* and *Candida albicans*.

Research software

The MatLab toolbox now available from Cambridge Control Solutions is designed to allow **access to the NAG Foundation Library** and an extensive set of numerical routines (*Reader Service No. 103*). The toolbox has been developed to help ease Fortran users into the MatLab technical computing environment, providing them with comprehensive numerical analysis capabilities and a wide range of powerful new tools, such as boundary value problem solvers. The toolbox contains more than 200 MatLab files, providing interactive access to the 250 routines in the NAG library. These cover optimization, ordinary and partial differential equations, quadrature, statistics and a wide range of other mathematical functions. New functions

perform tasks such as solving boundary value problems, performing adaptive, single- and multi-dimensional quadrature, fitting to curves and surfaces and directly accessing LAPACK algorithms for solving linear equations. Matrix solvers allow quantitative feedback theory controllers to be applied, even with Robust and Mu, which solve highly complex techniques.

Perkin-Elmer's new AutoAssembler software is designed to be an affordable, easy-to-use **package that automatically performs labour-intensive DNA sequence-assembly tasks** (*Reader Service No. 104*). It utilizes a Macintosh platform to execute its automated, batch-mode sequence clean-up and feature identification; a 'point-and-click' command assembles the sequences for easy viewing and editing of results. This product is said to be ideal for



AutoAssembler sequence analysis from Perkin-Elmer.

research performing small- to medium-size sequencing projects, but can be upgraded for large-scale projects. The manufacturer states that powerful algorithms determine the optimum sequence order, resulting in a precise consensus sequence. Results can be viewed graphically and interchanged as project layouts, bar and sequence alignments, and aligned, multiple, four-colour electropherograms.

A new introduction from B/T SciTech is the GelCypher **gel analysis software** for analysis of gels for quantitative PCR, Southern, northern, and western blots, differential display PCR and RFLPs (*Reader Service No. 105*). The package is designed for use with the SpeedLight Platinum gel-documentation system and is said to be intuitive, to simplify gel analysis, to improve ambiguity and to increase repeatability. The program is designed for a Windows interface, using simple commands to correct for smearing, slants, smiles and background variation, so that results are accurate and reproducible. Images can be stored within a gallery and

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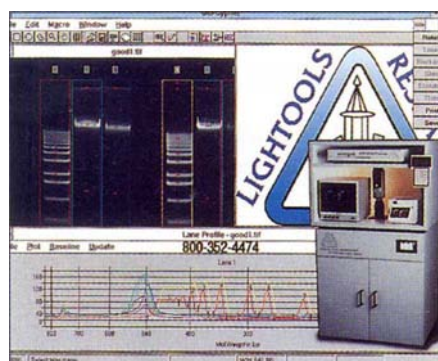


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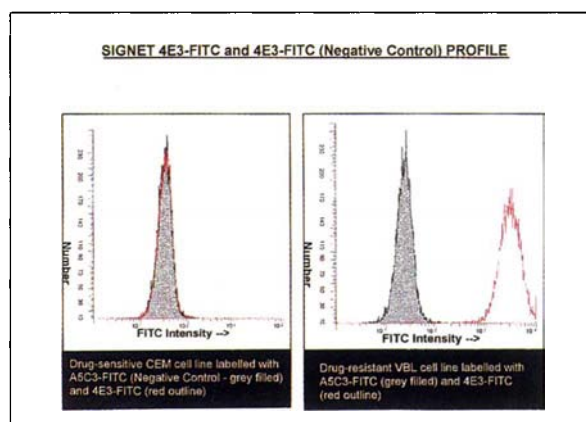
Serotec has recently launched a new range of **high-specificity immunological reagents** against crucial components of the intracellular signal transduction and transcription apparatus (*Reader Service No. 106*). The manufacturer states that this new range, together with its other complementary product, forms the

basis of a significant range of products specially designed to help researchers study aspects of cellular functions, from cell surfaces to the nucleus. Included in this range are three new antibodies to different forms of nitric oxide synthase.

Signet Laboratories introduces **directly conjugated, primary antibodies** for the investigation of P-glycoprotein (p170) and multidrug resistance (MDR) in flow cytometry applications (*Reader Service No. 107*). Multidrug resistance is often associated with the failure of tumour cells to respond to chemotherapeutic agents. Monoclonal antibody clone 4E3, a specific external epitope marker of p170, is now available conjugated to biotin, fluorescein or phycoerythrin. The company also offers isotype-matched, negative-control antibodies conjugated at the same ratios as the 4E3 primary antibody to ensure consistent results and to assist in test validation. The 4E3 antibody is available puri-

fied and conjugated for direct labelling of human-derived specimens. It is not cytotoxic, which allows for multiple research applications with live cell cultures. Detailed flow cytometry protocols, representative histograms and reference literature are available upon request.

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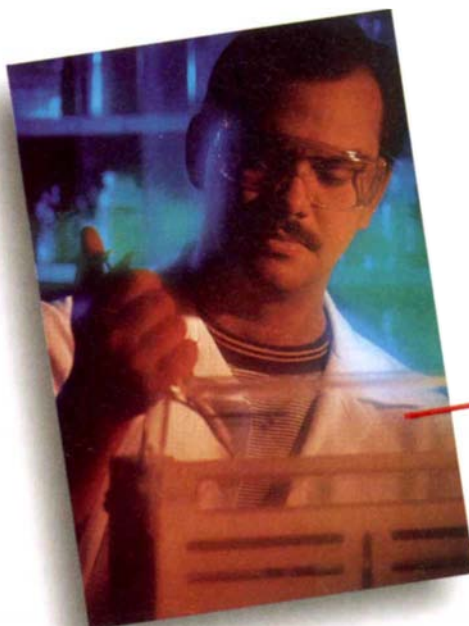


Signet Laboratories' antibodies for P-glycoprotein.

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- Prior to Sigma, worked five years in a molecular biology group in the pharmaceutical industry.
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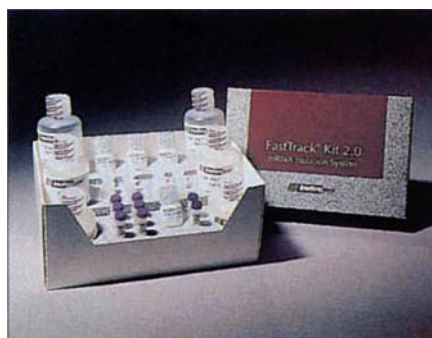


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vation (*Reader Service No. 108*). Levels of peripheral, blood-soluble CD27 (sCD27) have been found to increase in patients suffering from a variety of immunopathological diseases, which suggests that measurement can serve as a marker for T-cell activation *in vivo*. CD27 is a lymphocyte-specific member of a growing TNF-receptor family of membrane-bound and soluble cytokines. The sCD27 can be found in the supernatants of activated lymphocytes with expected values below 350 units per millilitre. The ELISA kit contains all the necessary components, including coating antibody, biotin-labelled antibody, IIRP-labelled streptavidin, standard, dilution buffer, three microtitre plates and a protocol. The manufacturer states that the sensitivity is 1 unit per millilitre and the intra- and interassay variations are less than 10 per cent. It is said that the kit contains sufficient reagents to perform 288 tests and that the assay can be performed in about four hours after overnight coating.

mRNA isolation

The FastTrack 2.0 mRNA isolation kit from Invitrogen offers the high yields of quality mRNA without the need for total RNA isolation (*Reader Service No. 109*). The kit utilizes premeasured oligo(dT) cellulose for the effective purification of mRNA direct from



Invitrogen's FastTrack 2.0 mRNA kit.

tissues, cells or plants. The isolated mRNA is said to be intact, pure and suitable for cDNA synthesis, RT-PCR, northern blots and oocyte microinjection.

Dynabeads mRNA Direct kit from Dynal provides a simple, 15-minute procedure for the **direct isolation of mRNA**, which can then be used as a template for RT-PCR (*Reader Service No. 110*). The manufacturer states that this kit has been tested successfully for direct isolation and detection of low-abundance mRNA from paraffin-embedded tissue that is more than ten years old. These paraffin-embedded lung tissues were obtained from post-mortem biopsies of

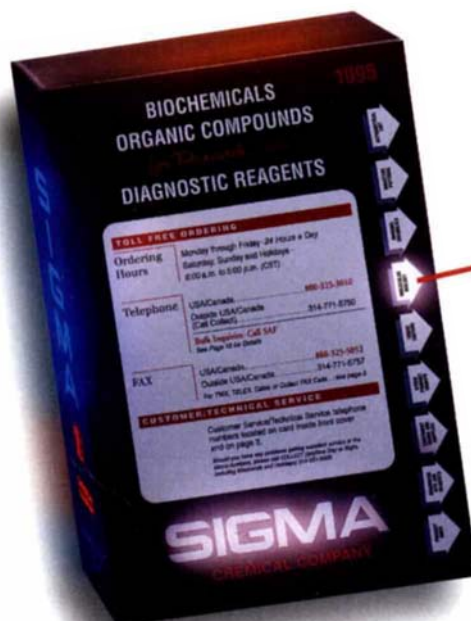
patients who died from toxic oil syndrome. This method is said to have detected not only β -actin mRNA, but also less abundant transcripts, such as IL-2, IL-4 and IL-5. The ability to obtain mRNAs from paraffin-embedded tissues that have been stored for more than ten years, gives the opportunity of studying and resolving processes of unknown aetiology after extended periods while benefitting from current knowledge.

Molecular biology

BioWhittaker has launched AccuGene, a complete line of ready-to-use, certified pure **molecular biology solutions** in convenient 500-ml and 1-l packaging (*Reader Service No. 111*). The company states that these products are manufactured in FDA-certified facilities, using research-grade reagents and ultrapure USP injectable water in their tissue culture media — large batch sizes are said to ensure purity and consistency for each bottle. Included in this range are TBE and TAE buffers, denaturing solution, EDTA, neutralizing solution, high-purity water and a variety of other commonly used solutions, which are available in 8-l and 18-l 'easy-spout' containers.

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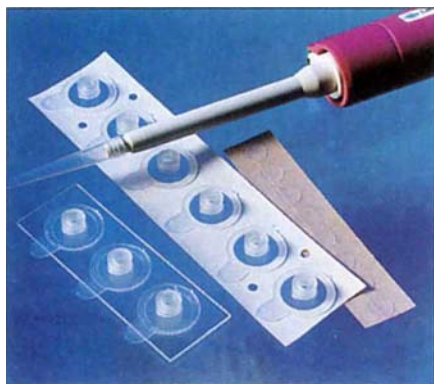
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slide-based *in situ* experiments and to prevent evaporation during thermal cycling (Reader Service No. 112). When used with the OmniSlide system, a total of 60 simultaneous, standardized *in situ* amplification reactions can be performed. This self-adhesive chamber is designed to eliminate the need for time-consuming methods of coverslip sealing, such as the use of nail polish and glue. Up to three SlideSeal chambers



Hybaid's OmniSlide with SlideSeal.

can be positioned on a slide, allowing test and control samples to be run side by side. After heat-bonding the chamber to a slide, creating a water-tight 15-mm diameter sample area, and adding up to 75 µl of amplification reagents, a completely vapour-tight seal is effected by a unique plastic filter. These two seals ensure the water does not evaporate during thermal cycling, maintaining the concentration of reaction buffers and stopping the sample from drying out.

Epicentre Technologies' new Retrotherm RT thermostable reverse transcriptase has both RNA-dependent and DNA-dependent polymerase activities (Reader Service No. 113). It is said to allow for both first- and second-strand cDNA synthesis in a single tube and in a single buffer. Isolated from a thermophilic bacterium, Retrotherm RT has been designed for optimal activity at temperatures above 65 °C. The high reaction temperatures obtainable with this enzyme allow for DNA synthesis from RNA templates with stable secondary structures. This protocol allows both strands to be synthesized

without the need to perform a buffer exchange or a chelation step.

Novagen offers the **M13mp18 cloning vector** in two ligation-ready formats (Reader Service No. 114). This single-stranded, filamentous bacteriophage vector permits blue and/or clear screening, enabling plaques containing inserts to be identified visually. The blunt-ended cloning kit contains *Sma*I- digested, dephosphorylated cloning vector DNA. A T-vector version of the linearized vector contains single 3' T-overhangs for direct cloning of fragments having single 3' dA-overhangs. Both configurations of the vector are available with pretested competent cell and ligation components, which are said to be rigorously tested for optimal cloning efficiency with minimal background.

Balancing acts

YMC has introduced the MJ series **precision balances** (Reader Service No. 115). The manufacturer states that these balances have been designed to be affordable and constructed with sophisticated quality control at each manufacturing step to ensure the products will give reliable performance for many years. The MJ series offers resolution as high as 0.001 g with capacities of 310 g, 510 g, 3,100 g and 6,200 g. These balances are controlled by a customized microcomputer, which was developed to have enough capacity for advanced internal software. These units include per cent function, counting, ten different measure units and an RS-232 interface.

Composite Rotor manufactures **lightweight, carbon-fibre, composite rotors** for high-, super- and ultra-speed centrifuges (Reader Service No. 116). The product line includes 48 rotor models that are compatible with Beckman, DuPont, Sorvall, Jouan, Kontron, Hitachi, as well as several other centrifuge manufacturers. The company says that the carbon-fibre composition of these rotors ensures that they will not corrode, have half the weight of comparable metal rotors, have faster acceleration and deceleration, include a full seven-year warranty, and have tube capacities from 1.5 ml to 500 ml. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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